

STATEMENT

Some of the experiments described in Paper
III and VI were carried out jointly with Dr. R.V. Jordan.
The electron microscopic preparations described in Paper
IV were made by Mr. R. Hill. The remainder of the work described in this
Thesis was carried out by the author.

STUDIES ON DELAYED-TYPE HYPERSENSITIVITY

Thesis submitted to The Australian National
University for the degree of
Doctor of Philosophy

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August, 1963.



TABLE OF CONTENTSSTATEMENT

Some of the experiments described in Paper III and VI were carried out jointly with Dr. S.V. Boyden. The electron microscopic preparations described in Paper IV were made by Mr. R.J. North. The histological preparations described in Paper VI were made by Mr. R. Hill. The remainder of the work described in this Thesis was done by the candidate.

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TABLE OF CONTENTS

	Page
A. INTRODUCTORY REVIEW:	
THE NATURE OF DELAYED-TYPE HYPERSENSITIVITY	
I. Introduction	1
II. Manifestations of Delayed-Type Hypersensitivity	9
Cutaneous reactions	
Other local tissue reactions	
Systemic reactions	
Passive transfer of hypersensitivity	
III. Phenomena related to Delayed-Type Hypersensitivity	16
Cellular immunity to infection	
Homograft rejection	
Auto-immune diseases	
Reactions to bacterial endotoxins	
IV. The Induction of Delayed-Type Hypersensitivity	28
Infection	
Homografts	
Tissue extracts and auto-immune diseases	
Contact sensitivity	
Pure protein antigens	
Other antigens	

	Antigenic specificity	
	The role and mode of action of adjuvants	
V.	Cellular reactions Associated with Delayed-Type Hypersensitivity	42
	Cellular reactions to antigen <u>in vivo</u>	
	Cellular reactions in tissue culture	
	Reactions of cells in suspension	
	Cellular uptake of antigens	
	Other cellular reactions <u>in vitro</u>	
VI.	The Modification of Delayed-Type Hypersensitivity Reactions	53
	Immunological unresponsiveness	
	Immunological enhancement	
	Pharmacological agents	
	Immunological deficiency diseases	
	Other factors	
VII.	Conclusion	64
B.	THE CUTANEOUS REACTIVITY OF GUINEA PIGS TO PURE PROTEIN ANTIGENS	
	Paper I. A Critical Evaluation of Methods for the Induction of Delayed-Type Hypersensitivity to Pure Proteins	66
	Paper II. The Effects of Different Routes of Injection of Antigen and Adjuvant	98

C. REACTIONS TO ANTIGEN OF PERITONEAL CELLS OF
GUINEA PIGS WITH DELAYED-TYPE HYPERSENSITIVITY

Paper III. The Loss of Macrophages from Peritoneal Exudates following the injection of Antigen into Guinea Pigs with Delayed-Type Hypersensitivity	109
Paper IV. The Fate of Peritoneal Macrophages after the Injection of Antigen into Guinea Pigs with Delayed-Type Hypersensitivity	134
Paper V. The Effects of Anticoagulants and other Drugs on Cellular and Cutaneous Reactions to Antigen in Guinea Pigs with Delayed-Type Hypersensitivity	157
Paper VI. The Uptake of Antigen <u>in vitro</u> by Peritoneal Cells of Normal and Hypersensitive Guinea Pigs	190

D. CONCLUDING DISCUSSION

I. Introduction	209
II. The Induction of Delayed-Type Hypersensitivity	209
III. The Mechanism of Delayed-Type Hypersensitivity Reactions	213
IV. The Interaction of Cellular and Humoral Factors in Delayed-Type Hypersensitivity and Homograft Rejection	222
V. Conclusion	225
Acknowledgements	228
References	229

It has long been recognized that recovery from an infectious disease is frequently followed by immunity to that disease. The practice of variolation or artificial infection of humans with material taken from the victims of small pox was widely used in Asia and Asia Minor from the 11th Century A.D. in the expectation (or hope) that a mild disease would result, leaving the survivor immune to small pox. In the late 18th Century

A.

INTRODUCTORY REVIEW

THE NATURE OF DELAYED-TYPE HYPERSENSITIVITY

Edward Jenner observed that people who had previously been exposed to cow pox and who later contracted small pox were not infected. He then showed that artificial infection with cow pox was also followed by immunity to small pox and he introduced the practice of "vaccination".

From the time of Pasteur on, the main stream of immunological investigation was concerned with the induction of immunity to infectious diseases, first by the use of vaccines of live attenuated organisms and later by the use of killed organisms. The discovery that plasma from man or animal could be used to protect against infection led to the discovery of antibodies and the function of phagocytes. The discovery of delayed hypersensitivity led to the discovery of cell-mediated immunity. The discovery of the nature of delayed hypersensitivity led to the discovery of the nature of the immune response.

It has long been recognized that recovery from an infectious disease is frequently followed by immunity to that disease. The practice of variolation or artificial infection of humans with material taken from the victims of small pox was widely used in Asia and Asia Minor from the 11th Century A.D. in the expectation (or hope) that a mild disease would result, leaving the survivor immune to small pox. In the late 18th Century Jenner observed that variolation was frequently unsuccessful in country people who had previously been exposed to cow pox and that the belief was prevalent among dairy workers that infection with cow pox protected them against small pox. He then showed that artificial infection with cow pox was also followed by immunity to small pox and he introduced the practice of "vaccination".

From the time of Pasteur on, the main stream of immunological investigation was concerned with the induction of immunity to infectious disease, at first by the use of vaccines of live attenuated organisms and later by the use of killed organisms. The discovery of bactericidal substances in the serum of man and animals by Buchner and Pfeiffer, and of the function of phagocytic cells by Metchnikoff led to some conflict between proponents of cellular and of humoral theories of immunity. These

were temporarily resolved by the demonstrations by Wright and by Denys that serum factors (opsonins) played an important part in the phagocytosis of micro-organisms and by Neufeld and Rimpau that the amounts of these opsonins in serum were increased after immunization.

It was soon discovered that not all responses to antigens in immune animals were necessarily beneficial. Portier and Richet (1902) found that dogs which had recovered after one injection of the poison of an actinian reacted very violently to a second intravenous injection, though the symptoms were not those of poisoning. They called this reaction anaphylaxis. Richet went on to demonstrate that anaphylaxis also occurred after repeated injections of non-toxic proteins. Arthus (1903) described the occurrence in rabbits of inflammation at the sites of repeated injections of horse serum. Soon after the first use in human patients of horse serum containing diphtheria and tetanus antitoxin, the occurrence of serum sickness in some patients was noted. In their classic monograph von Pirquet and Schick (1905) showed that the characteristic symptoms of fever, arthralgia, lymphadenitis and urticaria occurred at a fairly constant interval of 10 days after the injection of horse serum and were associated with the development of antibodies to the serum injected.

Meanwhile the tubercle bacillus had been discovered by Koch. He later found that the injection into tuberculous guinea pigs of old tuberculin (a concentrated filtrate derived from old cultures of tubercle bacilli) caused their death, although it was not toxic to normal animals even in much larger quantities. Koch was primarily interested in the possibility that tuberculin could be used in the treatment of tuberculosis by stimulating the cellular reaction around foci of infection (Koch, 1891). This was a vain hope and other workers were more interested in the tuberculin reaction as an aid to diagnosis. Subcutaneous injections into tuberculous animals or humans were found to cause an intense local inflammatory reaction as well as focal reactions at the sites of tuberculous infection (Epstein, 1891; Escherich, 1892; both cited by von Pirquet, 1911). Intradermal injections were found to be safer and more reliable (Mantoux and Lemaire, 1909).

In 1911 von Pirquet coined the term "allergy" (from the Greek allos, altered and ergeia, activity), and considered that many reactions, such as the tuberculin reaction, the accelerated reaction to revaccination, the exanthem of measles, serum sickness and anaphylaxis, could be grouped together under this title and were manifestations of immunological reactions due to antibodies (von Pirquet,

1911).

This classification proved, however, to be too simple. Zinsser (1921) was the first to describe the cutaneous reaction to tuberculin in tuberculous guinea pigs as delayed and to distinguish it from the cutaneous reactions of guinea pigs to proteins to which they had been anaphylactically sensitized. Zinsser pointed out that the reactions of animals to antigens of the causative organisms after infection with Salmonella typhosa, Brucella abortus and Malleomyces mallei resembled the tuberculin reaction rather than the early skin reactions. Zinsser and Mueller (1925) further showed that early cutaneous reactivity could regularly be transferred to normal guinea pigs by injections of serum from sensitized animals, while delayed reactivity could be transferred only irregularly, if at all. They considered that delayed skin reactions were due to a cell-associated factor which was not easily, if at all, separable from the cells of hypersensitive animals. Zinsser's emphasis on the morphological distinction between early and delayed reactions, the failure of most attempts to transfer delayed reactivity with serum and the implication of a cell-associated factor in delayed reactions represented a fundamental change in the approach to the problems of tuberculin hypersensitivity. The distinction between the

two types of reactivity was further highlighted by the work of Opie (1924) showing that the intensity of skin reactions of the Arthus type in guinea pigs was directly proportional to the amount of precipitating antibody in the animals' sera. Further impetus was given to the concept of cell-associated factors in delayed-type hypersensitivity by the work of Rich and Lewis (1928, 1932). They reported that the cells of spleen explants from tuberculin-sensitive guinea pigs showed diminished motility and often died in the presence of tuberculin, whether these explants were cultured in plasma from normal animals or from hypersensitive animals; explants from normal animals cultured in plasma from hypersensitive animals were not affected by tuberculin.

A further indication of the importance of cell-associated as opposed to humoral factors in delayed reactions came from the work of Landsteiner and Chase (1942), who transferred contact sensitivity to simple chemicals (a type of sensitivity resembling tuberculin sensitivity) from sensitized to normal animals by means of mononuclear cells from sensitized guinea pigs. Later, Chase (1945) also succeeded in transferring delayed-type hypersensitivity to tuberculin from hypersensitive to normal animals by similar means. An early report of the successful transfer of tuberculin sensitivity to normal animals by means of cells

(Bail, 1910) seems to have gone largely unnoticed. In none of these cases had attempts to transfer sensitivity with serum been successful.

The possibility of a relationship between delayed-type hypersensitivity and immunity to infection was highlighted by studies showing that the macrophages of specifically immunized animals possessed a heightened power to destroy vaccinia virus (Beard and Rous, 1938), psittacosis virus (Meyer, 1941) and tubercle bacilli (Lurie, 1942). The association with cells of the factors responsible for both immunity and delayed-type hypersensitivity in some infectious diseases has been a stimulus to efforts to understand the mechanism of delayed-type hypersensitivity reactions.

An important step in this direction was taken when Freund and McDermott (1942) succeeded in inducing delayed-type hypersensitivity to horse serum by injecting the serum as an emulsion in paraffin oil containing killed tubercle bacilli. The use of adjuvants containing Mycobacteria has allowed the study of delayed-type hypersensitivity to proteins other than those derived from micro-organisms.

Even more interesting uses for adjuvants have been found in the induction of tissue damage by the injection of tissue extracts in adjuvants. Iso-allergic encephalo-

myelitis was induced in this way by Freund, Stern and Pisani (1947) and by Kabat, Wolf and Bezer (1947). Interest in delayed-type hypersensitivity has been greatly stimulated by the close association between the development of these diseases and the development of delayed cutaneous reactivity to the tissue antigens in sensitized animals, by the fact that, like delayed-type hypersensitivity, these diseases can be induced in unsensitized animals by the injection of cells, but not serum, from sensitized animals, and by speculations that certain human diseases such as rheumatoid arthritis may have a basis in auto-immunity (Burnet, 1959).

A further stimulus to investigations of delayed-type hypersensitivity has come from interest in immunity to allogeneic tissue homografts. Like delayed-type hypersensitivity, homograft immunity has been regularly transmissible only by means of cells, not by serum from immunized animals. Furthermore, animals which have rejected skin homografts have also been found to have developed delayed cutaneous reactivity to antigens of the donor animal; and the histological features of homograft rejection closely resemble those of delayed skin reactions.

characterized by increased vascular permeability.

In the review which follows an attempt has been made to adhere to a standard terminology. Delayed-type hypersensitivity describes a state, the most characteristic feature of which is the ability of the animal to react to intradermally injected antigen with a delayed skin reaction. The term delayed cutaneous reactivity is used almost synonymously. "Delayed hypersensitivity" is not used, because the animals are in a state of hypersensitivity at all times and only the skin reactions are delayed. The term Arthus reaction is used for those skin reactions which are maximal 3 to 6 hours after the intradermal injection of antigen and subside rapidly thereafter. Animals which react in this way are said to be in a state of Arthus hypersensitivity or to possess Arthus skin reactivity. The eponymous term is used for convenience and in accordance with custom, despite the fact that these reactions in guinea pigs are in some ways dissimilar from the reactions in rabbits which Arthus originally described. The term immediate skin reaction is used for those reactions which are apparent within a few minutes of injection, maximal in an hour or less, subside very rapidly and are chiefly characterized by increased vascular permeability.

(Raffel, Arnold, Dukes and Huang, 1949). Although they

are less easily elicited, delayed skin reactions also occur

II. MANIFESTATIONS OF DELAYED-TYPE HYPERSENSITIVITY

Cutaneous reactions

The most characteristic manifestation of delayed-type hypersensitivity is the delayed reaction to intradermally injected antigen. The appearance of such a reaction was described by Zinsser (1921) when he distinguished "the delayed type of tuberculin reaction" as "one in which there is no immediate effect, but in which within 4, 5, or more hours, a swelling becomes apparent which in the course of 12 to 24 hours results in a swollen edematous area of varying intensity, often with a central necrotic spot and, occasionally, hemorrhage. This reaction may not reach its highest development until about 48 hours after the injection, and is accompanied by distinct signs of inflammation and some cell death". This description was given of reactions to tuberculin in tuberculous guinea pigs, but applies equally well to delayed skin reactions to other bacterial products after infection of guinea pigs, rabbits and man (Raffel, 1954), simple chemicals after the induction of contact sensitivity in guinea pigs and man (Chase, 1954) and pure proteins in guinea pigs sensitized with the antigen in adjuvant (Raffel, Arnaud, Dukes and Huang, 1949). Although they are less easily elicited, delayed skin reactions also occur

in rats (Flax and Waksman, 1962), mice (Crowle, 1959a) and chickens (Szenberg and Warner, 1962). Microscopically, delayed reactions are characterized by the accumulation of macrophages and lymphocytes, particularly in perivascular areas. Variable polymorph infiltration has been observed, mostly in the early stages of the reactions or later if necrosis of the skin has occurred. Contrary to Zinsser's original description, oedema is not a notable feature of delayed reactions (Dienes and Mallory, 1932; Gersh and Black, 1939; Gell and Hinde, 1951; Flax and Waksman, 1962). In contrast, Arthus reactions are characterized by intense polymorph infiltration and oedema (Humphrey, 1955).

Other local tissue reactions

Reactions similar to delayed skin reactions in their time-course and histology have been elicited in the corneas of rabbits and guinea pigs. Delayed corneal reactivity to the appropriate antigen has been observed to follow infection with tubercle bacilli (Holley, 1935; Rich and Follis, 1940), and pneumococci (Julianelle, 1930 b) and the injection of proteins in complete adjuvant (Raffel, 1950; Salvin and Gregg, 1961).

In mice, local delayed inflammatory reactions have been observed after the injection of antigens into

the foot pads of mice infected with tubercle bacilli (Gray and Jennings, 1955; Crowle, 1959 c) and Listeria monocytogenes (Mackaness and Ackerman, 1962).

Microscopically, these reactions also resemble delayed skin reactions in other species.

The injection of tuberculin into the thigh muscles of tuberculous mice has been reported to result in a delayed local inflammatory response (O'Grady, 1957; Crowle, 1959 c).

Systemic reactions

The systemic administration of antigens to animals with delayed-type hypersensitivity has characteristic effects. There is commonly a transient febrile response 1 to 2 hours after injection; this may be followed by profound shock with lowered body temperature and blood pressure and sometimes death, particularly if a large dose of antigen is injected. There is also a marked monocytopenia and lymphopenia in the peripheral blood. Such effects have been observed to follow the injection of tuberculin into tuberculous humans and guinea pigs and rabbits (Friis, 1955; Johanovský, 1959 a) and tuberculous rats and mice (Hehre and Freund, 1939; Kirchheimer and Malkiel, 1953; Crowle, 1960). They have also been observed after the injection of antigens into guinea pigs with delayed-type hypersensitivity to non-bacterial proteins

(Dienes, 1931; Salvin, 1962).

In humans, guinea pigs and mice with tuberculous lesions, systemic administration of tuberculin is also followed by acute inflammation around those lesions (Steidl and Heise, 1931; Platt, 1954; Crowle, 1959 b) - the so-called focal tuberculin reaction.

Passive transfer of hypersensitivity

In guinea pigs, rats, rabbits and mice delayed cutaneous reactivity to bacterial antigens, pure proteins, tissue antigens and simple chemicals can be conferred on normal animals by injections of cells derived from lymphoid tissue or from peritoneal exudates (Landsteiner and Chase, 1942; Chase, 1945; Crowle, 1959 a; Najarian and Feldman, 1961; Boughton and Schild, 1962; Flax and Waksman, 1962). Large numbers of cells (10^8 - 10^9) are required and the reactions are frequently much weaker in the recipient than in the donor. Reactivity occurs whether the cells are injected systemically or at the same site as the antigen. Cells killed by heating or freezing are ineffective (Chase, 1953; Metaxas and Metaxas-Buhler, 1948). Cells enclosed in cell-impermeable Millipore chambers have also been reported to be ineffective (Najarian and Feldman, 1962). Passively transferred hypersensitivity is not of long duration unless the donor

and the recipient animal are histocompatible, as the donor cells are treated as a homograft and destroyed (Bauer and Stone, 1961; Warwick, Archer and Good, 1962).

Systemic reactivity to antigens in delayed-type hypersensitivity has also been transferred to normal rabbits and guinea pigs by means of cells from donors with delayed-type hypersensitivity (Kirchheimer, Weiser and Liew, 1949; Johanovský, 1959 a).

In man, delayed-type hypersensitivity to microbial or tissue antigens has been transferred to normal individuals by means of peripheral blood leucocytes taken from hypersensitive donors. In contrast with the general experience with animals, Lawrence and his associates reported that disrupted leucocytes and extracts of leucocytes were as effective as intact leucocytes. The factor responsible (transfer factor) was resistant to treatment with DN ase, RN ase and trypsin. The transfer factor was released from cells when they were incubated with the specific antigen. This observation supports the contention that the transfer was not due to the injection of antigen, as also does the observation that hypersensitivity could not be transferred from man to monkeys or rabbits. This passive transfer system in man differs from those in animals in that the state of delayed-type hypersensitivity persisted for up to a year in the recipients (Lawrence,

1955, 1956, 1959 a, 1960; Lawrence and Pappenheimer, 1956, 1957; Rapaport, Lawrence, Millar, Pappagianis and Smith, 1960; Rapaport and Millar, 1959).

Insufficient numbers of experiments have been performed to allow an assessment of these findings. The necessity for a critical approach is emphasized by the facts that the release of transfer factor from cells incubated with antigen was demonstrated in only 2 experiments, with 1 donor and 6 recipients; and that cell free extracts prepared by centrifugation of disrupted leucocytes were used in only 3 pairs of donors and recipients. Some confirmation of these important findings has, however, been provided by Freedman, Fisher and Cooke (1957) using disrupted leucocytes and cell free extracts of leucocytes and by K. Jensen, Patnode, Townsley and Cummings (1962) using disrupted leucocytes.

Other types of hypersensitivity can also be passively transferred by means of cells, provided that these are capable of producing antibody (Chase, 1953). In animals and man lymph node cells are effective in transferring antibody producing capacity as well as delayed-type hypersensitivity (S. Harris, T.N. Harris, Ogburn and Farber, 1956; Good, Varco, Aust and Zak, 1957).

Other types of hypersensitivity (Arthus or immediate) are regularly transferable with serum from

sensitized donors (Ovary, 1958), but delayed-type hypersensitivity is irregularly if at all transferable in this way. Zinsser and Mueller (1925) elicited delayed skin reactions in the recipients of serum from tuberculous guinea pigs, but only in a very small proportion of the animals tested. Rauch and Favour (1960) described the passive transfer of delayed-type hypersensitivity to tuberculin by means of a plasma protein fraction (Cohn Fraction IV - 10) obtained from the sera of hypersensitive guinea pigs. The effect of this fraction was partly neutralized by gamma globulin in Fraction II. If a serum factor responsible for delayed-type hypersensitivity exists, its detection may well be hampered by the presence of inhibitors in other serum protein fractions.

III. PHENOMENA RELATED TO DELAYED-TYPE HYPERSENSITIVITY

Cellular immunity to infection

The early studies of Beard and Rous (1938), Lurie (1942) and Meyer (1941) suggesting a cellular rather than a humoral mechanism in immunity to certain diseases have been cited above. The idea that immunity to certain parasites, notably Brucella and Mycobacterium tuberculosis, might be entirely a function of macrophages led to a great deal of work with conflicting results (reviewed by Elberg, 1960) and no firm conclusion. For example, Suter (1953) reported that the multiplication of tubercle bacilli was partially or completely suppressed in cultures of macrophages from immunized guinea pigs and rabbits, compared with those from normal animals. Mackaness (1954 a,b) could find no inhibition of bacterial multiplication in the cells of immunized rabbits. He suggested that Suter's results could be due to crowding of macrophages in the cultures derived from immunized animals, as bacterial multiplication was less in crowded cultures of cells from normal animals than in sparse cultures. Recently, however, Mackaness (1962 a) has studied Listeria monocytogenes infection in mice and has shown that in mice which have recovered from infection a high degree of immunity exists, this immunity

being associated with changes in the properties of the macrophages. Macrophages from immune mice were highly resistant to infection with Listeria in vitro, whereas serum from these animals failed to confer immunity on normal mice and failed to influence the growth of Listeria in the spleens of normal mice, even when the bacteria were incubated with the serum in vitro before infection. The development of delayed-type hypersensitivity in mice infected with a sublethal dose of Listeria paralleled their development of resistance to the infection. In man and animals infected with vaccinia virus the existence of delayed-type hypersensitivity is associated with immunity to reinfection with vaccinia or small pox, even when antibodies are not detectable (Turk, Allison and Oxman, 1962; R.M. Friedman and Baron, 1961). The idea that the same change in macrophages may be responsible for both immunity and delayed-type hypersensitivity is an attractive one. Some findings have, however, been interpreted as strong evidence against it. Mackaness and Ackerman (1962) found that immunity to Listeria in mice declined more rapidly than did hypersensitivity. Rothschild, Friedenwald and Bernstein (1934) vaccinated guinea pigs with avirulent tubercle bacilli and desensitized some of the animals by repeated injections of tuberculin so that they failed to react to skin tests. On challenge with

virulent organisms these animals developed tuberculosis of the same degree of severity as did the controls. Similar results were reported by M. Gordon (1963) using Pasteurella pestis. Delayed-type hypersensitivity to tuberculin can be induced by injecting guinea pigs with tuberculin in complete adjuvant (containing tubercle bacillary wax), but this procedure results in no immunity to infection with virulent tubercle bacilli (Raffel, 1950). None of these findings, however, provides conclusive evidence against a very close relationship between the cellular changes responsible for the two states. It is possible that the antigens actually used to elicit delayed reactions are not the same as those against which protective mechanisms are developed and that were the right antigen to be used for skin testing a closer parallel might be observed. Williams (1960) found that delayed-type hypersensitivity was induced by the same extracts of tubercle bacilli as were effective in inducing immunity. Desensitizing procedures may simply immobilize reactive cells, so preventing them appearing at a skin test site, rather than changing their reactivity to the antigen. The question of the relationship between delayed-type hypersensitivity remains an open one.

Homograft rejection

Arguments have frequently been adduced linking

the mechanism of homograft rejection with that of delayed-type hypersensitivity (e.g. Lawrence, 1959 b). It has been repeatedly demonstrated that the ability to reject allogeneic homografts in accelerated fashion can be conferred upon normal animals by lymphoid cells from an animal which has already rejected a graft from the same or a genetically identical donor. Intact peripheral blood leucocytes (in man and animals) and disrupted leucocytes (in man) are also effective (Billingham, Silvers and D.B. Wilson, 1962; Lawrence, Rapaport, Converse and Tillett, 1960). Most workers have found that serum from sensitized animals does not have this effect (reviews by Snell, 1957; Brent, 1958). Homografts implanted in cell-impermeable Millipore chambers survive indefinitely even in sensitized mice (Algire, Weaver and Prehn, 1954). The cellular reaction in the bed of a homograft undergoing rejection bears a close resemblance to the cellular reaction in a delayed skin reaction (Waksman, 1960, 1963), the most notable feature being the perivascular accumulation of mononuclear cells (lymphocytes and macrophages). The rejection of skin homografts in guinea pigs and man is associated with the development, in the recipients, of delayed-type hypersensitivity to antigens of the donor. In guinea pigs and rabbits the reactivity of the recipient's lymphoid cells to antigens of the donor has

also been demonstrated by injecting these cells intradermally into the donor's skin. The resulting "transfer reaction" has the temporal and histological characteristics of a delayed-type hypersensitivity reaction (Brent, Brown and Medawar, 1959, 1962; Merrill, E.A. Friedman, R.E. Wilson and D.C. Marshall, 1961; Dvorak, Kosunen and Waksman, 1963). The attractive idea that similar mechanisms may determine delayed-type hypersensitivity and immunity to homografts remains, like the possible connection between immunity to infection and delayed-type hypersensitivity, unproven.

The picture is further complicated by the "white graft" phenomenon. It is usual for accelerated rejection of a second homograft to occur after an interval of some days during which it becomes vascularized. In certain circumstances, however, the second graft may, like a graft from a foreign species, not become vascularized at all, remaining white and undergoing rapid necrosis. This occurs in mice and rabbits if the second graft is placed very soon after the first has been rejected or if the recipients have been hyperimmunized with large doses of donor cells incorporated in Freund's complete adjuvant. White grafts have also been described in humans who have received a second graft within 14 days of the rejection of the first, or a succession of grafts from the same donor. Histologically,

the reaction in the graft bed resembles an Arthus reaction and the effect is passively transferable with serum from the hyperimmunized recipients (Stetson, 1959 a; Stetson and Demopoulos, 1958; Stetson and E. Jensen, 1960; E. Jensen and Stetson, 1961; Chutná, 1961; Chutná and Pokorná, 1961; D.C. Marshall, E.A. Friedman, Goldstein, Henry and Merrill, 1962).

The participation in "conventional" homograft rejection of factors not intimately associated with cells is also suggested by other work. Najarian and Feldman (1962) and Kretschmer and Pérez-Tamayo (1962) have reported accelerated rejection of skin homografts in mice and rabbits carrying cell-impermeable Millipore chambers filled with lymphoid cells from sensitized donors. They have suggested that a humoral antibody is liberated from the cells in the chambers and brings about homograft rejection. The possible roles of cell-associated and humoral factors in the rejection of homografts are subjects for considerably more experimentation and debate, particularly as the situation is still further complicated by the effects of some antibodies in prolonging the lives of homografts (immunological enhancement, discussed below).

These remarks apply mainly to homografts of solid tissues. The injection of dissociated cells, for example from bone marrow or lymph nodes, results in the

development of homograft immunity of this sort, but these cells are themselves destroyed almost invariably by humoral antibodies (T.N. Harris, S. Harris and Farber, 1961; Garver and Cole, 1961).

Auto-immune diseases

Close similarities also exist between delayed-type hypersensitivity and certain diseases induced in animals by immunization with their own or homologous tissue antigens. With a few exceptions, the induction of these diseases requires the use of adjuvants containing Mycobacteria (Freund, 1956) or substances derived therefrom (White, 1959); the active substances are identical with those effective in promoting the induction of delayed-type hypersensitivity to pure protein antigens. The development of "auto-immune" disease in experimental animals is accompanied by the appearance of antibodies and delayed cutaneous reactivity directed towards the antigens of the tissue used. The incidence and severity of the disease is paralleled by the degree of delayed cutaneous reactivity and not by the antibody titre (McMaster, Lerner and Exum, 1961; Miescher, Gorstein, Benacerraf and Gell, 1961). Both the disease and delayed cutaneous reactivity can be passively transferred in normal animals by the injection of lymphoid cells, but not serum, from

sensitized animals (Astrom and Waksman, 1962; Boughton and Schild, 1962). There is also a close similarity between the histological features of experimental autoimmune diseases and delayed skin reactions (Waksman and Adams, 1962; Flax, 1963). It is possible that the same fundamental change in macrophages and/or lymphocytes is responsible for the tissue damage and the delayed-type hypersensitivity, though this remains an attractive speculation only.

Reactions to bacterial endotoxins

Since the original work of Sanarelli (1924) and Schwartzman (1928) on the reactions of animals to the endotoxins of Gram-negative bacteria, these substances have been found to produce a great variety of effects when injected into animals or humans. These include the production of fever, lymphocytopenia, monocytopenia, polymorphonuclear leucocytosis, shock, abortion, an adjuvant effect on antibody formation and variations in resistance to infection which are paralleled by variations in the activity of the macrophages of the reticulo-endothelial system; there may also be hyperreactivity to epinephrine, intravascular agglutination of leucocytes and platelets, the production of a peculiar form of fibrinogen precipitable by heparin, thrombosis or the activation of fibrinolytic

mechanisms (see reviews by Rosen, 1961; Stetson, 1959 b, 1961; Thomas, 1954, 1959).

There has been much interest in the phenomenon of local tissue reactivity (local Schwartzman reaction) in which the site of an intradermal injection of endotoxin exhibits hemorrhagic necrosis when an intravenous injection of endotoxin is given after a suitable interval. Another reaction depending on the use of two suitably spaced injections of endotoxin is the generalized Schwartzman or Sanarelli-Schwartzman reaction, in which bilateral renal cortical necrosis due to intravascular thrombosis follows the second of two intravenous injections of endotoxin.

Some of these reactions resemble the reactions to antigen in animals with delayed-type hypersensitivity. The reactions to intradermal injections of endotoxin or erythrocytes coated with endotoxin are delayed (Stetson, 1955; Lee and Stetson, 1960; Sell and Braude, 1961). The systemic reactions, with fever and changes in the peripheral leucocyte counts, are very similar to systemic reactions in delayed-type hypersensitivity (Mechanic, Frei, Landy and W.W. Smith, 1962) while the features of generalized Schwartzman reaction can be reproduced by injections of tuberculin into rabbits with delayed-type hypersensitivity to tuberculin (Stetson, 1955).

The mediation by cells of at least some of the

biological effects of endotoxins is suggested by several lines of evidence. Increased resistance to bacterial infection occurring after the injection of endotoxin is associated with an increased capacity of macrophages to destroy bacteria in vivo or in vitro, independently of the increase in serum opsonic capacity which also occurs (Whitby and Rowley, 1959; Rowley, 1960). The hyperreactivity of rabbit aorta to epinephrine in vitro is dependent on the presence of blood cells in the perfusion medium (Gourzis, Hollenberg and Nickerson, 1961). The enhancing effect on antibody formation of endotoxin injected into rabbits at the same time as antigen is associated with greatly increased cellular proliferation in the spleen (Thorbecke, Asofsky, Hochwald and Siskind, 1962).

The suggestion has been made that many of the biological effects of endotoxins may occur because of the existence of "natural" delayed-type hypersensitivity to these substances, perhaps induced by the Gram-negative bacteria in the gut (Stetson, 1955). The similarities pointed out above and the diminished susceptibility to endotoxins of mice with few Gram-negative bacteria in the gut (Schaedler and Dubos, 1962) support this view.

There are, however, similarities between some effects of endotoxins and effects of antigens in the presence of humoral antibodies. Platelet and leucocyte

thrombi, which occur in Schwartzman reactions, also occur during Arthus and anaphylactic reactions. Platelet clumping can be induced in vitro by antigen-antibody complexes in the presence of complement (Stetson, 1951; Siqueira and R.A. Nelson, 1961). Antigen-antibody complexes can accelerate the coagulation of rabbit blood in vitro just as endotoxin can (Robbins and Stetson, 1959) and can induce a reaction similar to the generalized Schwartzman reaction in rabbits in vivo (Lee, 1963). Possible explanations of the relationship between cellular and humoral factors in the reactivity of animals to endotoxin are discussed again below in the light of further experiments in this laboratory. Some apparent conflicts may be due to the poorly defined chemical nature of endotoxins used in different experiments. These may be resolved when correlations can be established between biological activity and the chemical structure of purified endotoxins (Ribi, Haskins, Landy and Milner, 1961; Westphal, 1960).

Animals (mice, rabbits, guinea pigs) infected with tubercle bacilli or other organisms which induce delayed-type hypersensitivity have been shown to exhibit increased cutaneous and systemic reactivity to bacterial endotoxins. This occurs despite an increase in the activity of the reticulo-endothelial system (RES), as measured by its ability to remove foreign material from the blood.

Paradoxically, such an increase is, in other circumstances, usually associated with decreased susceptibility to endotoxin. Concomitant infection with Gram-negative bacteria has been excluded by Suter as a factor in this hyperreactivity, the mechanism of which remains unclear (Holzberger and Packalén, 1954; Box and Briggs, 1961; Johanovský, Vrána, Vejhora and Francová, 1961; Suter and Kirsanow, 1961; Suter, 1962; L.J. Berry, Smythe and Kolbye, 1962).

(Spink, 1956); mycotic infections (Sullivan, 1961); bacterial infections, for example, with diphtheria bacilli (Pappenheimer and Lawrence, 1948; Ehr, Pappenheimer and Yanada, 1957), streptococci (Moore, 1956) and pneumococci (Allison, 1930 a); infections with viruses, for example, vaccinia (W. Smith, 1932; Turk, Allison and Owen, 1962), lymphogranuloma venereum (Frei, 1925, 1938), mumps (Enders, Cohen and Kane, 1945) and herpes simplex (Rose and Melloy, 1947); and syphilis in human and rabbit (Rich, Shoenberger and Turner, 1933).

Dead organisms are much less effective than live organisms in inducing delayed-type hypersensitivity. Killed tubercle bacilli induce only feeble delayed-type hypersensitivity to tuberculin unless they are suspended in paraffin oil (Boyden, 1937 a).

The substances effective in eliciting delayed

IV. THE INDUCTION OF DELAYED-TYPE HYPERSENSITIVITY

Infection

It seems probable that in man and experimental animals many, if not all, infections lead to the development of delayed-type hypersensitivity. Delayed-type hypersensitivity has been reported to follow: chronic bacterial infections, such as tuberculosis and brucellosis (Spink, 1956); mycotic infections (Salvin, 1963); acute bacterial infections, for example, with diphtheria bacilli (Pappenheimer and Lawrence, 1948; Uhr, Pappenheimer and Yoneda, 1957), streptococci (Moen, 1936) and pneumococci (Julianelle, 1930 a); infections with viruses, for example, vaccinia (W. Smith, 1932; Turk, Allison and Oxman, 1962), lymphogranuloma venereum (Frei, 1925, 1938), mumps (Enders, Cohen and Kane, 1945) and herpes simplex (Rose and Molloy, 1947); and syphilis in humans but not rabbits (Rich, Chesney and Turner, 1933).

Dead organisms are much less effective than live organisms in inducing delayed-type hypersensitivity. Killed tubercle bacilli induce only feeble delayed-type hypersensitivity to tuberculin unless they are suspended in paraffin oil (Boyden, 1957 a).

The substances effective in eliciting delayed

skin reactions are usually stated to be proteins (Raffel, 1954). This has in most cases been a deduction rather than an experimentally proven fact. In the case of tuberculosis, however, good evidence exists that the effective substances are proteins and not polysaccharides, although humoral antibodies react with the polysaccharides (Boyden and Sorkin, 1956). The substances which elicit the skin reactions are usually referred to as antigens, though it is unusual for these substances to be effective alone in inducing delayed-type hypersensitivity.

Homografts

"Conventional" homograft immunity (i.e. the accelerated rejection of a second allogeneic homograft) can be regularly induced: (1) by a previous graft of tissue (in man and all experimental animals); (2) by the previous injection of lymphoid cells or nucleated blood cells by intradermal, subcutaneous, intravenous or intraperitoneal routes (in guinea pigs and mice; intravenous injections of rabbits with cells actually prolongs the life of a subsequent graft (Billingham, Brent and Mitchison, 1957; Billingham, 1957)); (3) by the injection of certain cell extracts (in mice, guinea pigs and rabbits (Billingham, Brent and Medawar, 1958; Medawar, 1959)) or platelets (in guinea pigs and rabbits (R.A.

Nelson, 1962; Ebbe, Baldini and Dameshek, 1962)).

Most of the studies on the chemical nature of transplantation antigens have been made using mice of inbred strains. The development of homograft immunity is accompanied by the formation of humoral antibodies and the antigens determining the induction of both responses are determined by the same genes in these animals. This fact has been utilized in studies of the chemical nature of the antigens and the assays of antigens in cell extracts have mostly been made by serological methods (Snell, 1957; Davies and Hutchison, 1961; Brent, Medawar and Ruszkiewicz, 1961). The antigens characterized in this way appear to be lipoproteins (Davies, 1962) and not, as was previously thought, deoxyribonucleoproteins (Billingham, Brent and Medawar, 1956) or mucoproteins (Hildemann and Medawar, 1959). It is debatable whether these antigens are identical with those which induce homograft immunity. In discussing this question Kandutsch (1961) has pointed out that the injection of preparations which induce the formation of antibodies may prolong the lives of homografts by immunological enhancement instead of inducing accelerated rejection. There is, nevertheless, no doubt that crude extracts of cells can induce immunity to homografts and that these extracts are also effective both in inducing and eliciting delayed-type hypersensitivity (Billingham, Brent

and Medawar, 1958; Brent, Brown and Medawar, 1958, 1959, 1962). Delayed corneal reactions can also be elicited by donor cell extracts in guinea pigs which have rejected skin homografts (R.F. Smith and Weiser, 1961).

So far as the available evidence goes, it seems that only those procedures which induce homograft immunity also induce delayed-type hypersensitivity. Furthermore, as with delayed-type hypersensitivity to other antigens, the mode of sensitization rather than the nature of the antigen may determine whether the result of immunization is delayed-type hypersensitivity, humoral antibody formation or both.

Tissue extracts and auto-immune diseases

There is general agreement that the development of auto-immune disease in experimental animals injected with autologous or homologous tissue extracts occurs regularly only when the extracts are incorporated in adjuvants containing Mycobacteria (reviews by Freund, 1956; Waksman, 1962) or substances derived from Mycobacteria. In the latter case these substances are the same as those which promote the development of delayed-type hypersensitivity to foreign proteins (White, 1959). Much less commonly these diseases can be induced in animals without the use of Mycobacterial adjuvants. This has been done in monkeys,

by means of repeated injections of tissue extracts (Rivers, Sprunt and G.P. Berry, 1933). In guinea pigs "allergic" encephalomyelitis can rarely be induced unless Mycobacteria are used (Paterson and Bell, 1962). In rats, sensitization without Mycobacteria is more often effective than in guinea pigs; a high incidence of "allergic" encephalomyelitis in rats so sensitized appears, however, to be a characteristic only of certain strains (Levine and Wenk, 1961). Comparative studies of the incidence of delayed cutaneous reactivity and disease in animals sensitized without Mycobacteria would be of interest.

The chemical nature of the antigens responsible for the induction of experimental auto-immune diseases has not been well studied. Thyroglobulin and picrylated thyroglobulin are effective in the induction of thyroiditis (Flaz, Jankovic and Sell, 1963; Miescher et al. 1961) but in most other cases crude homogenates of tissues have been used. A curious correlation exists between the presence in rat brain extracts of encephalitogenic substance and thromboplastin (Svet-Moldavsky and Kriegshaber, 1963).

In man, disseminated encephalomyelitis is a rare sequel to some immunization procedures, notably rabies vaccination. Some diseases of man which have been considered to have a basis in auto-immunity, such as rheumatoid arthritis, systemic lupus erythematosus and

Hashimoto's thyroiditis seem frequently to arise spontaneously. Sometimes, however, there is a history of virus infection preceding the development of thyroiditis. Rheumatic fever, sometimes considered to be an "auto-immune" disease is usually preceded by infection with haemolytic streptococci. If any of these diseases are actually due to auto-immunity delayed-type hypersensitivity rather than humoral antibodies may be responsible. The nature of the antigens against which auto-immunity is directed might well be investigated with this in mind. There are, however, few reports of such investigations. Delayed skin reactions to autologous leucocytes and to human arterial antigens in patients with systemic lupus erythematosus have been described (E.A. Friedman, Bardawil, Merrill and Hanau, 1960; Vaccari, Baldini and Fontana, 1962). Attempts to implicate delayed-type hypersensitivity to antigens of human nervous tissue as a pathogenetic factor in multiple sclerosis have been unsuccessful (Stauffer and Waksman, 1954; Böhme, Paal, W. Kersten and H. Kersten, 1963).

In some strains of rats and guinea pigs a disease characterized by disseminated granulomas and arthritis can be induced by the injection of Mycobacterial adjuvants alone (Chase, 1959 b; Pearson, Waksman and Sharp, 1961; Pearson and Wood, 1963). This may prove to

be a more useful model for human disease than the experimental "auto-immune" diseases affecting specific organs or tissues.

Contact sensitivity

Contact sensitivity to certain simple chemicals, for example, dinitrofluorobenzene and picryl chloride, can be induced in guinea pigs by intradermal injections of the chemical alone, or by injection of conjugates of the chemical with foreign or homologous proteins by any route when the conjugates are incorporated in Mycobacterial adjuvants. Sensitization by intradermal injections of what are in effect haptens is believed to depend on their conjugation in vivo with proteins of skin. Some of these procedures frequently also induce the formation of humoral antibodies and Arthus hypersensitivity directed towards the hapten. Delayed reactions may be elicited by the application of the chemical to the skin or by the intradermal injection of the conjugates (Chase, 1954; Eisen, 1959; Benacerraf and Gell, 1959 a, b; Salvin and R.F. Smith, 1961).

Pure protein antigens

Delayed-type hypersensitivity to pure protein antigens can regularly be induced in guinea pigs and rabbits by injections of the proteins emulsified in

Mycobacterial adjuvants. Unlike the pure delayed reactivity following infection, this is usually accompanied by some Arthus hypersensitivity. Certain procedures have been described as effective when adjuvants lacking Mycobacteria (paraffin oil plus an emulsifying agent) are used. These include the use of minute quantities of antigen (Salvin, 1958); complexes of antigen with excess antibody (Uhr, Salvin and Pappenheimer, 1957; Sell and Weigle, 1959); denatured proteins (Gell and Benacerraf, 1959; R.T. McCluskey, Miller and Benacerraf, 1962) and proteins coupled with haptens (Benacerraf and Gell, 1959 a). The reactions elicited after sensitization in these ways differ morphologically from classical delayed reactions, as seen, for example, in the tuberculin reaction, and the duration of the state of hypersensitivity is short. Work reported in this thesis supports the contention of Raffel and Newel (1958) that these reactions are of a different type from those of true delayed-type hypersensitivity. They may be classed with the transient "delayed" reactions described by Jones and Mote (1934), and Mote and Jones (1936), which occurred in humans immunized with foreign serum.

It is possible that delayed-type hypersensitivity to protein antigens may be induced by intracorneal injections, in the absence of Mycobacterial adjuvants

(Parks, Leibowitz and Maumenee, 1962; Leibowitz and Parks, 1963).

Other antigens

It has been noted above that delayed skin reactions cannot be elicited by non-protein substances from infecting micro-organisms. Janković and Waksman (1962) reported the induction of delayed-type hypersensitivity to purified human blood group substances in guinea pigs. It is probable, however, that this was directed against the protein and not against the polysaccharide moiety of these substances (Loewi and Holborow, 1962).

In guinea pigs, delayed-type hypersensitivity has been induced to artificial antigens (dinitrophenyl-polylysine and dinitrophenyl copolymer of lysine and glutamic acid) injected in complete adjuvants (Kantor, Ojeda and Benacerraf, 1963). In man, repeated intradermal injections of ethylene oxide-treated human serum without adjuvants have been reported to induce delayed-type hypersensitivity to this substance (Maurer, 1961).

Antigenic specificity

A notable characteristic of humoral antibodies formed after immunization is their exquisite specificity. This is probably not true of natural antibodies (R.A. Nelson,

1958). The lack of purity of the bacterial extracts used to elicit delayed skin reactions after infection of animals and man has not permitted the detection of a similar degree of specificity for these antigens. In tuberculosis it is known that tuberculins derived from different species or strains of Mycobacteria show a low degree of specificity when used to skin test animals infected with the same or different Mycobacteria (Boyden and Sorkin, 1956). On the other hand, tuberculin sensitive animals exhibit a greater sensitivity to endotoxins of Gram-negative bacteria than do normal animals. This may be simply due to the presence in Mycobacteria of similar endotoxins acting as an antigenic stimulus.

The use of pure protein antigens and conjugates of simple chemical haptens with these proteins has allowed some conclusions to be drawn concerning the antigenic specificity of delayed-type hypersensitivity. Studies on animals sensitized with proteins and hapten-protein conjugates in adjuvants containing Mycobacteria have shown that the delayed-type hypersensitivity is directed towards the protein and is as specific for the protein as is humoral antibody; chemical treatments which reduce the ability of the protein to react with precipitating antisera or to induce anaphylaxis also reduce its ability to elicit

delayed skin reactions. Arthus hypersensitivity, but not delayed-type hypersensitivity, is directed towards the hapten. Contact sensitivity to the hapten is absent or weak in animals sensitized with a conjugate of the hapten with a foreign protein, but is strong when the hapten is conjugated with homologous protein. This suggests that contact sensitivity is directed against the animal's own protein modified in vivo by conjugation with the hapten (Benacerraf and Gell, 1959 b; Gell and Benacerraf, 1961; Salvin and R.F. Smith, 1961; Benacerraf and Levine, 1962; Leskowitz, 1962). Similar conclusions regarding the specificity of Arthus and delayed reactions have been drawn from tests of corneal sensitivity in sensitized animals (Salvin and Gregg, 1961).

Salvin (1962) also studied the systemic reactions to antigens in guinea pigs sensitized with protein-hapten conjugates. The early febrile response was elicited only by the conjugate or the protein used for sensitization and not by a conjugate of the hapten with an unrelated protein. The delayed shock was elicited by a conjugate of the hapten with an unrelated protein, suggesting that this component of the systemic reaction was not due to delayed-type hypersensitivity to the protein.

Suggestions have been made that delayed-type hypersensitivity is a stage in humoral antibody production

and is directed against a larger portion of the protein molecule than are humoral antibodies (e.g. Gell and Benacerraf, 1959). These have been based on experiments in which sensitivity was induced without the aid of Mycobacterial adjuvants. It is doubtful whether the hypersensitivity so induced is actually of delayed-type. Furthermore, delayed-type and Arthus hypersensitivity can co-exist in the same animal, notably in guinea pigs sensitized with proteins in complete adjuvant.

The role and mode of action of adjuvants

Reference has frequently been made in the preceding sections to the necessity for the presence of Mycobacteria or their derivatives in the sensitizing inoculum used to induce delayed-type hypersensitivity or experimental "auto-immune" disease. The effective substances are present in Mycobacterium tuberculosis, M. butyricum, M. phlei, Nocardia asteroides, and a wax fraction derived from Mycobacteria (Raffel et al., 1949; Freund, 1956; White, 1959).

The mode of action of these substances is unknown. Both whole organisms and their active fractions stimulate the formation of a granuloma at the site of injection, proliferation of macrophages throughout the body, increased plasma cell responses, increased antibody production and delayed-type hypersensitivity after the

injection of antigen (White, Coons and Connolly, 1955).

It is not known how these effects are produced or what the interrelationship among them, causal or otherwise, may be. An intimate relation between the antigen and the adjuvant emulsion is necessary. Raffel et al.

(1949) found that if the antigen was injected into the site of injection of the adjuvant emulsion after an interval of 2 hours, delayed-type hypersensitivity was induced, but if the antigen injection was made after 24 hours delayed-type hypersensitivity was not induced.

They also found that delayed-type hypersensitivity was not induced when the wax was added to a watery emulsion of the antigen instead of to a water-in-oil emulsion.

Stone and Freund (1959) found that injections of adjuvant at sites close to those of antigen injections were effective, but injections at distant sites were ineffective.

The injection of adjuvant some time before the injection of an emulsion of antigen in adjuvant depresses the response to the antigen, perhaps because of an immunological reaction against the components of the adjuvants (Kies and Alvord, 1958; Janković, 1962). Some of these findings are slightly at variance with those of Dienes (1929) who induced delayed-type hypersensitivity to egg white by injecting it into large granulomas resulting from two previous injections of tubercle bacilli. The reason for

this discrepancy is not known.

V. CELL Only the vaguest explanation can be offered for the mode of action of adjuvants: that somehow their incorporation in the sensitizing inoculum alters the way in which the antigen is "processed" by cells (perhaps by macrophages) before it reaches immunologically competent cells, and that this alteration determines the development of delayed-type hypersensitivity.

reaction, a fact too often forgotten in the numerous studies of the cytotoxic effects of antigens on cells of animals with delayed-type hypersensitivity. In guinea pigs sensitized passively with tritiated thyroglobulin-labelled cells from sensitive donors, the cells which accumulate at the sites of delayed skin reactions are largely, if not entirely derived from the recipient animal. This suggests that either the transferred cells synthesize some substance which is then taken up by the recipient's cells, or only a few "sensitized" cells are required to react with the antigen to cause the subsequent accumulation of donor and recipient macrophages at the site of the reaction (Najarian and Feldman, 1961; Turk, 1962; H.T. McCluskey, Benaseraf and J.W. McCluskey, 1963).

Studies of the effects on cells of antigens injected by other routes have failed to throw light on the mechanism of this apparently simple reaction. Mollet (1921)

V. CELLULAR REACTIONS ASSOCIATED WITH DELAYED-TYPE
HYPERSENSITIVITY

Cellular reactions to antigen in vivo

Intradermal injection of antigen into animals with delayed-type hypersensitivity is followed by the accumulation of macrophages at the site. Death of or damage to these cells is not characteristic of the delayed reaction, a fact too often forgotten in the numerous studies of the cytotoxic effects of antigens on cells of animals with delayed-type hypersensitivity. In guinea pigs sensitized passively with tritiated thymidine-labelled cells from sensitive donors, the cells which accumulate at the sites of delayed skin reactions are largely, if not entirely derived from the recipient animal. This suggests that either the transferred cells synthesize some substance which is then taken up by the recipients' cells, or only a few "sensitized" cells are required to react with the antigen to cause the subsequent accumulation of donor and recipient macrophages at the site of the reaction (Najarian and Feldman, 1961; Turk, 1962; R.T. McCluskey, Benacerraf and J.W. McCluskey, 1963).

Studies of the effects on cells of antigens injected by other routes have failed to throw light on the mechanism of this apparently simple reaction. Holst (1921)

found that the exudate cells harvested some time after the intraperitoneal injection of tuberculin into hypersensitive guinea pigs were mostly dead, whereas the cells from normal animals injected with tuberculin were mostly alive. Stewart, Long and Bradley (1926) made similar observations on pleural exudates of normal and hypersensitive guinea pigs injected with tuberculin 24 hours previously. The doses of tuberculin used were very large and the observed cytotoxic effect could well have been due to the large numbers of polymorphonuclear leucocytes appearing in the exudates of the hypersensitive animals injected with tuberculin; many of these cells, which have a short life span, could have died within 24 hours. Dreisbach, Snell and Speirs (1956) examined the cell populations after the injection of allogeneic tissue extracts into the peritoneal cavities of normal mice and mice which had previously rejected tumour homografts. The differences were slight, the previously injected animals showing higher contents of eosinophils and macrophages 4 to 10 days after injection. The eosinophilia was probably a result of the interaction of antigens with humoral antibodies (Speirs, Speirs and Jansen, 1961; Litt, 1961). These studies bear a closer relation to the observation on skin reactions, though the changes occurred over a longer period of time; they offer no clue to the mechanism of

the reactions.

Cellular reactions in tissue culture

Rich and Lewis (1928, 1932) described the "toxic" effect of tuberculin on cultures of explants of spleens from tuberculous guinea pigs. This effect was manifested as an inhibition of migration of cells from the explants and death of the cells occurring usually after 24-48 hours' incubation. Macrophages and fibroblasts were said to be affected. These changes were seen in explants of spleens from tuberculous guinea pigs in the presence of tuberculin and either normal plasma or plasma from tuberculous animals; normal cells in the presence of tuberculin and plasma from tuberculous animals were unaffected. The dose of tuberculin used to elicit these effects was, however, much larger than the usual skin test dose and was close to that which was also toxic for the cells of normal animals. Similar observations have been made by many workers using specific antigen and tissues from animals with a variety of infections¹, including tuberculosis (Aronson, 1931; Fabrizio, 1952; Paas, Flick, Kapral and Rudd, 1961), haemolytic streptococcal infections (Moen, 1936), and brucellosis (Heilman, Howard and Carpenter, 1958).

¹See reviews by Favour (1957) and Waksman (1958).

Epithelial cells of hypersensitive animals, for example from liver (Buckley, Buckley and Keeve, 1951) and kidney (Packalén, Tunçman and Wasserman, 1959) but not cornea (May and Weiser, 1956) have been reported to be similarly affected by antigen in tissue culture. Carpenter, Fukuda and Heiskell (1962) used monolayer cultures of cells derived from the spleen and found that the cells from animals hypersensitive to Brucella antigens were damaged or died to a greater extent in the presence of those antigens than did cells from normal animals.

Inhibition by antigen of the migration of cells from the buffy coat of blood cultured in vitro has also been used as an index of the supposedly cytotoxic effect of antigen for the cells of animals with delayed-type hypersensitivity. This has been demonstrated in man and animals with delayed-type hypersensitivity to tuberculin (O'Neill and Favour, 1955; Hall and Scherago, 1957; Packalén, Wasserman and Weibull, 1962), histoplasmin (Johnson and Scherago, 1960) and mumps virus (Wasserman and Packalén, 1962). The migration of peritoneal exudate cells from hypersensitive animals similarly cultured in capillary tubes has also been reported to be inhibited by the specific antigen (George and Vaughan, 1962; David, El-Askari, Lawrence and Thomas, 1963).

The relationship between these phenomena and the

reactions of cells to antigens in vivo is not clear. The "cytotoxic" effects have usually been obtained with doses of antigen far larger than those required to elicit a skin reaction. The cells which are damaged include those which have not been shown to respond to antigen in vivo, such as hepatic and renal cells. There is, moreover, little correlation between the results of skin tests and the results of tissue culture experiments (Stinebring, Flick and Pomales-Lebrán, 1958). It has been suggested that the toxicity of antigens may be merely a reflection of the increased susceptibility to noxious stimuli in general of cells from hypersensitive animals (Lasfargues, Boquet and Delaunay, 1947). This suggestion receives some support from the work of Packalen and his colleagues. They showed that the migration of cultured cells from animals with delayed-type hypersensitivity to tuberculin or mumps virus was inhibited not only by the specific antigen but by other antigens, by bacterial endotoxins and by dextran. Non-specific inhibition of migration also occurred in cultures from normal animals but was more marked in cultures from hypersensitive animals (Packalén, et al. 1959; Tuncman and Packalén, 1959; Wasserman and Packalén, 1959, 1962; Wasserman, 1962).

Not all investigators have found antigens to be

toxic in vitro for the cells of animals with delayed-type hypersensitivity. Juhasz-Schäffer (1928) found a greater proliferation of cells in explants from the organs of hypersensitive guinea pigs cultured in the presence of tuberculin than in those cultured without tuberculin. Waksman and Matoltsy (1958) reported similar findings with peritoneal exudate cells. Švejcar and Johanovský (1961 c) found that cultured spleen and peritoneal exudate cells from hypersensitive guinea pigs survived better in the presence of antigen than in its absence. Mackaness (1962 b) found that a large number of mitoses occurred in cultures of macrophages when these cells were taken from the peritoneal cavities of animals injected intravenously some time previously with antigens of L. monocytogenes to which they were hypersensitive. Pearmain, Lycette and Fitzgerald (1963), W.H. Marshall and Roberts (1963) and Elves, Roath and Israëls (1963) have all reported recently that extensive mitosis can be induced in cultures of peripheral blood leucocytes from tuberculin-sensitive humans when these cells are cultured in the presence of tuberculin. These findings are more obviously related to events occurring in the intact animal with delayed-type hypersensitivity, in which the macrophages of the reticulo-endothelial system are known to be more active and numerous, especially after antigenic challenge (Myrvik, Leake and Oshima, 1962).

So many workers have demonstrated an inhibitory effect of antigen on the migration of cells cultured from animals with delayed-type hypersensitivity that one cannot dismiss the findings. It seems unjustified, however, to attribute this to a toxic effect of antigen. In studies in which the conditions of culture were carefully examined and controlled, Marks (1958) showed that the primary effect of tuberculin on cultured cells from hypersensitive guinea pigs was to inhibit their migration. When cultural conditions were adverse the cells died as a result of being confined to the explant. Some of the work reported in this thesis suggests that the change induced in macrophages of hypersensitive guinea pigs by the injection of antigen is an increased tendency to adhere to one another and to other cells. This may also occur in vitro and may explain the decreased mobility of cells cultured in the presence of antigen; the question is further discussed below (Part D, III).

The reactions of cells in suspension

Favour (1947) described an effect of tuberculin on the peripheral blood lymphocytes of tuberculous mice and guinea pigs, whereby the lymphocytes were lysed in the presence of tuberculin. This effect was, however, apparently due to an interaction among cells, antigen,

humoral antibody and complement (J.M. Miller, Favour, B.A. Wilson and Umbarger, 1949; J.M. Miller, Vaughan and Favour, 1949) and was not related to skin reactivity or tissue culture sensitivity (Waksman, 1953 a).

Leucocytolysis has been reported to occur when peripheral blood leucocytes from Brucella infected guinea pigs are incubated in vitro with antigens of Brucella (Feeley and Pickett, 1962). A similar effect occurs when rabbit blood containing humoral antibody is incubated with the specific antigen (Waksman, 1953 b). The loss of cells from suspension may be due to their adherence to the walls of the tube in which they are incubated and the implication of cytotoxicity of antigen seems not to be justified. The relationship of this phenomenon to delayed-type hypersensitivity is also doubtful.

Johanovský (1959 b) described a method for determining the viability of cells which seemed to allow the detection of a specific cytotoxic effect of antigen in vitro on the cells of hypersensitive animals. Subsequent experiments (Švejcar and Johanovský, 1961 a, b, c) showed that the effect was quite non-specific.

Cellular uptake of antigens

The implication of cell-associated factors in the reactions of delayed-type hypersensitivity has led to a

widespread assumption that these reactions are due to "cell-bound antibody", implying an antibody of "conventional" type fixed in some way onto the surface of cells. That such substances exist in other situations is known. Plasma cells in the act of producing humoral antibody frequently have some associated with their surfaces (Mäkelä and Nossal, 1961 a, b). The serum of rabbits immunized with soluble antigens (without complete adjuvant) contains cytophilic antibodies, i.e. humoral antibodies which can become attached to spleen cells and confer upon them an ability to take up antigen (Boyden and Sorkin, 1960, 1961).

Attempts to demonstrate a specific affinity for tuberculin in vivo or in vitro of cells from animals with delayed-type hypersensitivity to tuberculin have met with little success. Tuberculin appeared to be taken up with equal avidity by cells from both normal and hypersensitive animals (Ritts and Favour, 1955; Turk, 1960). Steffen and Rosak (1963) showed by indirect means that the lymph node cells of guinea pigs with delayed-type hypersensitivity to ovalbumin had a slightly greater affinity than normal cells for the antigen. Berrian and Brent (1958) showed, also indirectly, that lymphoid cells from mice sensitized to allogeneic tissue had some affinity for the antigens of that tissue. None of these experiments indicated the high specific affinity for antigen which might be expected from the

specificity and even violence of the reactions to antigen in vivo. Possible reasons for these failures and the possible relationship of cytophilic antibody to delayed-type hypersensitivity are discussed again below in the light of experiments described in this thesis.

Other cellular reactions in vitro

Other reactions have been described which implicate specific cell-associated factors in some phenomena related to delayed-type hypersensitivity. R.A. Nelson (1962) reported that lymph node cells from guinea pigs which had rejected skin homografts adhered to human erythrocytes in vitro in the presence of complement; this was thought to indicate, by means of immune adherence, the presence of antibody bound to the surface of these cells because of a cross reaction between antigens of the graft donor and recipient. Lymphocytes from sensitized animals have been reported to adhere to and destroy allogeneic cells in tissue culture (Weaver, 1958; Govaerts, 1960; Rosenau and Moon, 1961). Blood leucocytes from patients with ulcerative colitis, a disease which may be due to auto-immunity, were found to cause increased liberation of intracellular constituents from foetal colon cells in culture. This effect was not shown in the absence of complement and was not produced by humoral antibodies reacting with the cells even in the presence of complement

(Broberger and Perlmann, 1963; Perlmann and Broberger, 1963).

THE MODIFICATION OF DELAYED-TYPE HYPERSENSITIVITY REACTIONS

Immunological unresponsiveness

Animals injected with an antigen before or shortly after birth are frequently unable to respond immunologically to a subsequent challenge with that antigen. This occurs after the injection of allogeneic cells, as shown by failure to reject a subsequent skin graft; and of foreign protein antigens, as shown by the failure of the injected animal to develop an allergic reaction (e.g. Medawar, 1950; R.T. Smith and Bridges, 1958; Sercarz and Cohen, 1959). Immunological unresponsiveness to microbial antigens injected in early life has been less easy to demonstrate. Weiss (1958) found that guinea pigs injected in utero with tuberculin or dead tubercle bacilli (BCG) showed diminished delayed-type hypersensitivity to tuberculin after subsequent challenge with BCG, compared with similarly challenged animals which had not been injected in utero. This diminution was not apparent when live BCG organisms were used either for the initial injection or for the challenge. Flick and Pinsus (1963) found that rabbits could be made tolerant to vaccinia virus by an injection of inactivated vaccinia virus soon after birth. This was shown by the inability of rabbits to develop

VI. THE MODIFICATION OF DELAYED-TYPE HYPERSENSITIVITY REACTIONS

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delayed-type hypersensitivity or humoral antibody or to resist infection on a subsequent challenge with live virus. Tolerance induced in guinea pigs by injections of foreign protein shortly after birth has been shown to depress not only the formation of humoral antibody but also the induction of delayed-type hypersensitivity by subsequent injections of the protein in Freund's complete adjuvant (Humphrey and Turk, 1961; Turk and Humphrey, 1961). Tolerance to contact sensitizing agents has been demonstrated in the offspring of female guinea pigs injected during pregnancy with the agents (Harber, Rosenthal and Baer, 1962).

Immunological unresponsiveness has been induced in adult animals by injections of large amounts of polysaccharide or protein antigens (Felton, 1949; Dixon and Maurer, 1955). This is usually referred to as paralysis to distinguish it from tolerance induced in early life; the distinction is probably artificial. Paralysed animals also fail to develop antibody when challenged with the antigen (Sercarz and Coons, 1959). Adult guinea pigs injected with large doses of serum from allogeneic donors fail to develop delayed-type hypersensitivity when challenged with an injection of serum in complete adjuvant (J. Gordon, 1962). Guinea pigs fed by intragastric tube with contact sensitizing chemicals

fail to develop contact sensitivity to those chemicals after challenge (Chase, 1959 a; Coe and Salvin, 1963). Partial or complete unresponsiveness to allogeneic tissue antigens can also be induced when these are injected into adult animals treated with agents, such as 6-mercaptopurine, which destroy immunologically competent tissue; this situation is more akin to that in neonatal animals injected with antigen (Schwartz, 1959; McLaren, 1961). The "tolerance" induced in adult animals by repeated injections of allogeneic tissue antigens may be due to immunological enhancement rather than paralysis (discussed below).

Desensitization of animals with delayed-type hypersensitivity to bacterial antigens is a phenomenon of a different sort; the failure of cells to accumulate at the skin test site is not accompanied by a failure of cellular immunity (M. Gordon, 1963).

Immunological enhancement

In mice previously injected with extracts of allogeneic tissue the growth and survival of tumor homografts from donors of the same allogeneic strain is frequently promoted, sometimes to a degree that leads to the death of the host. Sera from mice so treated show high titres of humoral antibodies to antigens of the donor

strain and will passively transfer this effect to other mice of the recipient strain, as also will antisera prepared in foreign species. This is the phenomenon of immunological enhancement of tumour grafts, which has been defined by Kaliss (1958) as the progressive growth of tumour homografts produced by specific antiserum in the host. Enhancement of allogenic homografts of other tissues has also been reported, for example, of skin grafts in mice (Brent and Medawar, 1962) and guinea pigs (D.S. Nelson, 1962) and of ovarian homografts in rats (Parkes, 1958). Enhancement of homografts of normal tissues is rarely as marked as enhancement of tumour homografts. It is possible that the prolonged survivals of skin grafts after repeated grafting (Prehn, 1961), multiple injections of allogeneic spleen cells (Shapiro, Martinez, Smith and Good, 1961), intravenous injections of dissociated epidermal cells (Billingham, 1957) and injections of large quantities of platelets (R.A. Nelson, 1962) were also due to enhancement by humoral antibodies, though this was not investigated.

The way in which certain humoral antibodies promote the survival of allogeneic grafts is not known. The phenomenon is the more puzzling when one recalls that in certain circumstances humoral antibodies can destroy grafts. Sometimes the same serum can cause the destruction

of tumour homografts when injected in large doses and enhancement when injected in small doses (Kaliss, 1958; Phillips and Stetson, 1962). Snell, Winn, Stimpfling and Parker (1960) suggested that the cellular immune response was depressed in animals possessing enhancing antibodies in their sera. Feldman and Globerson (1960) showed, however, that mice bearing tumour homografts passively enhanced by injected antisera still produced an intense cellular immune reaction to the grafts, indicated by the accelerated rejection of a second non-enhanced graft after excision of the first. Cock (1962 a, b) found that homografts of allogeneic testis and ovary in fowls could survive for long periods in the face of an intense cellular reaction as shown by histological studies of the rejection of skin grafts from the same allogeneic donor. That enhancement might be operative was shown by the significant prolongation of survival of these skin grafts and by the development of humoral antibodies to antigens of the donor animal. It is not clear from these or from other experiments whether a cellular reaction of normal intensity actually occurs in the beds of enhanced homografts at the time at which non-enhanced grafts would be rejected.

Phenomena analogous to homograft enhancement occur in other situations. Boyden (1957 b) showed that

injections of tuberculoprotein into guinea pigs caused the formation of humoral antibodies and the development of Arthus hypersensitivity but depressed the development of delayed-type hypersensitivity after subsequent sensitization with live tubercle bacilli (BCG). Shaw, Fahlberg, Kies and Alvord (1960) described suppression of the development of experimental allergic encephalitis in guinea pigs injected with encephalitogenic proteins before challenge with those proteins in complete adjuvant. Paterson and Harwin (1963) reported similar findings in rats and showed that serum containing antibodies to rat brain passively transferred this protection to other animals. Janković and Flax (1963) found that the development of auto-immune thyroiditis in guinea pigs injected with thyroid tissue extract in complete adjuvant was delayed in animals previously injected with thyroid extract alone. It is well known too that tolerance to the pyrogenic effects of endotoxins occurs in rabbits repeatedly injected with endotoxins. This effect can be passively transferred to other animals with serum from tolerant animals. The nature of the serum factor is not known with certainty but it may be an antibody to endotoxin. Its formation occurs at the same time as that at which tolerant rabbits react to intradermal injections of endotoxin with Arthus instead of delayed reactions (Lee and Stetson, 1960;

Greisman, Carozza and Hills, 1963).

Pharmacological agents

Adrenocortical hormones (cortisone, hydrocortisone and their acetates) have been reported to inhibit delayed skin reactions to tuberculin in hypersensitive humans, guinea pigs, rabbits and mice (J.B. Long and Favour, 1950; D.A. Long and Miles, 1954; Germuth, Ottinger and Oyama, 1951; S. Harris and T.N. Harris, 1950; Crowle, 1960). The degree of inhibition was dependent on the initial degree of sensitization, the duration of treatment and the dose used; guinea pigs required relatively larger doses than other animals. The febrile response to systemically injected antigen was also inhibited in some experiments with rabbits and guinea pigs (S. Harris and T.N. Harris, 1950) but not in others, in which smaller doses of hydrocortisone were given to guinea pigs (Salvin, 1962). The local Schwartzman reaction in rabbits was also found to be inhibited (Humphrey, 1951). Baldrige and Kligman (1951) and Jeter and Seeborn (1952) found that cortisone did not inhibit contact sensitivity in guinea pigs, but they used relatively small doses and/or short courses of treatment. Cortisone also inhibits the rejection of allogeneic skin grafts in guinea pigs (Sparrow, 1954) and the development of experimental allergic encephalomyelitis in monkeys (Kabat, Wolf and Bezer, 1952).

The mode of action of adrenocortical hormones is not understood. The phagocytic activity of mouse macrophages in vivo is not affected by cortisone (Gell and Hinde, 1953). The migration of leucocytes in vitro is inhibited by hydrocortisone (Ketchel, Favour and Sturgis, 1958). Hydrocortisone also inhibits the destruction of allogeneic cells in tissue culture by lymphocytes of sensitized mice, though it does not inhibit the attachment of the lymphocytes to the target cells (Rosenau and Moon, 1962). Some experiments bearing on the possible mode of action of cortisone in delayed-type hypersensitivity are described below.

Aminomethylpteroylglutamic acid, a cytotoxic drug, has been reported to inhibit the development of delayed-type hypersensitivity to foreign proteins in guinea pigs, but not the manifestations of previously induced hypersensitivity (R.M. Friedman, Buckler and Baron, 1961). The inhibition by another cytotoxic drug, 6-mercaptopurine, of the rejection of homografts (Schwartz and Dameshek, 1960; Meeker, Condie, Good and Varco, 1960) and the development of experimental allergic encephalitis (Hoyer, Good and Condie, 1962) is probably also related to the induction rather than the expression of hypersensitivity. On the other hand, treatment with agents which grossly reduce the number of circulating leucocytes in the blood, such as

X-irradiation, benzol and nitrogen mustard, inhibits the manifestations of previously induced hypersensitivity (Packalén, 1952; Pepys, 1955).

Massive doses of the antihistaminic drug Phenergan have been reported to inhibit delayed skin reactions in guinea pigs to tuberculin (Pepys, 1955) but not to allogeneic tissue antigens (Brent, Brown and Medawar, 1962). Smaller doses of Phenergan have been reported not to inhibit cutaneous or systemic reactions to antigen in hypersensitive guinea pigs (Salvin, 1962).

The release of "hypersensitivity pyrogen" from mixtures of antigen and extracts of cells of hypersensitive guinea pigs is inhibited by protease inhibitors (Johanovský and Škvařil, 1962).

Immunological deficiency diseases

In patients with Hodgkin's disease or related lymphomatous conditions the ability to develop delayed-type hypersensitivity, contact sensitivity and homograft immunity is depressed. The depression varies with the activity of the disease. The formation of humoral antibodies is not affected. These patients frequently cannot develop delayed skin reactions to antigen even after the injection of leucocytes from hypersensitive donors (D.G. Miller, Lizardo and Snyderman, 1961; Sokal and Primikiri, 1961;

Aisenberg, 1962; Lamb, Pilney, Kelly and Good, 1962; Good, Kelly Rötstein and Varco, 1962).

Patients with sarcoidosis also have an impaired ability to develop delayed-type hypersensitivity but a normal ability to form humoral antibodies. Although the lesions of the disease resemble tubercles, patients remain tuberculin negative even when they have active tuberculosis or have been vaccinated with BCG (Lemming, 1940; Carnes and Raffel, 1949). Delayed-type hypersensitivity can be passively transferred to patients with sarcoidosis by leucocytes from hypersensitive donors (Good et al., 1962).

In patients with agammaglobulinaemia the ability to form humoral antibodies is grossly impaired. The ability to develop delayed-type hypersensitivity, to reject skin grafts and to develop rheumatoid disease is unimpaired (Good, Bridges, Zak and Pappenheimer, 1959; Good et al., 1962).

Other factors

Delayed skin reactions in hypersensitive humans and animals are depressed by scurvy, hypothyroidism, diabetes and intercurrent infection; measles and measles vaccines are particularly effective (Rich, 1951; Thompson, 1961; Mueller and Kies, 1962; Mellman and Wetton, 1963).

Thymectomy of newborn animals grossly depresses

all immunological functions in later life, including the ability to develop delayed-type hypersensitivity, homograft immunity and experimental auto-immune diseases (J.F.A.P. Miller, 1961, 1962; Arnason, Janković and Waksman, 1962).

(1)

The nature of the changes which lead to the altered reactivity of cells (macrophages and lymphocytes) in delayed-type hypersensitivity. The existence of "cell-bound antibodies" has frequently been postulated but never proven. Even if such substances can be shown to exist, other problems remain, for example, by which kind of cell they are carried, whether by all the cells of a given type or only by some, and whether they are synthesized by the cells carrying them or are synthesized elsewhere and then bound to these cells.

(2)

The nature of the forces attracting cells to

VII. CONCLUSION

Delayed-type hypersensitivity is a very common sequel to a variety of antigenic stimuli. The interest of the investigator in delayed-type hypersensitivity is stimulated not only by the inherent fascination of a poorly understood phenomenon, but also by the close association between these reactions and immunity to some infections, the rejection of allogeneic homografts, and auto-immune diseases.

The major unsolved problems in this field are:-

- (1) The nature of the changes which lead to the altered reactivity of cells (macrophages and lymphocytes) in delayed-type hypersensitivity. The existence of "cell-bound antibodies" has frequently been postulated but never proven. Even if such substances can be shown to exist, other problems remain, for example, by which kind of cell they are carried, whether by all the cells of a given type or only by some, and whether they are synthesized by the cells carrying them or are synthesized elsewhere and then bound to these cells.
- (2) The nature of the forces attracting cells to

and holding them at the sites of delayed skin reactions, the beds of homografts undergoing rejection and the sites of tissue damage in auto-immune diseases.

- (3) The mechanisms underlying the induction of delayed-type hypersensitivity as opposed to the induction of humoral antibody formation and Arthus hypersensitivity. Delayed-type hypersensitivity is usually accompanied by humoral antibody formation, but the reverse is not true. The inhibition by preformed humoral antibody of some manifestations of delayed-type hypersensitivity also seems to indicate a dichotomy between the two responses.
- (4) The absence of a reliable test for delayed-type hypersensitivity other than the skin test. Solutions to the other major problems will probably become available much more readily when such a test is devised.

The experiments described in this thesis offer clues to the answers to some of these problems.

SUMMARY

The reactions to intradermal injections of antigen following the sensitization of guinea-pigs with pure proteins were observed and compared with the classical tuberculin reaction. Native, heat denatured

B. THE CUTANEOUS REACTIVITY OF GUINEA PIGS TO PURE PROTEIN ANTIGENS

in incomplete adjuvant or in complete adjuvant (containing *Mycobacterium*). The animals were skin tested 7 and 14

days afterwards and in some cases later. There developed

with antigen in complete adjuvant a delayed hypersensitivity

PAPER I.

resembling tuberculin hypersensitivity in that the skin

reactions were maximal 24 to 36 hours after testing and

persisted for 48 to 72 hours. The reactions 7 days after

sensitization were weak but were usually unaccompanied

by an early (Arthus) component. The reactions 14 days

after sensitization were stronger but there was usually

an Arthus component. The delayed component was not

greatly inhibited by repeated skin tests. In contrast,

animals sensitized with antigens in incomplete adjuvant

did not respond to skin tests with tuberculin-type

reactions. When tested 7 days after sensitization, some

animals had skin reactions which were delayed in onset

SUMMARY

The reactions to intradermal injections of antigen following the sensitization of guinea-pigs with pure proteins were observed and compared with the classical tuberculin reaction. Native, heat denatured and picrylated albumins and albumins complexed with excess antibody were injected into the foot pads, either in incomplete adjuvant or in complete adjuvant (containing Mycobacteria). The animals were skin tested 7 and 14 days afterwards and in some cases later. Those injected with antigen in complete adjuvant developed hypersensitivity resembling tuberculin hypersensitivity in that the skin reactions were maximal 24 to 30 hours after testing and persisted for 48 to 72 hours. The reactions 7 days after sensitization were weak but were usually unaccompanied by an early (Arthus) component. The reactions 14 days after sensitization were stronger but there was usually an Arthus component. The delayed component was not greatly inhibited by repeated skin tests. In contrast, animals sensitized with antigens in incomplete adjuvant did not respond to skin tests with tuberculin-type reactions. When tested 7 days after sensitization, some animals had skin reactions which were delayed in onset

but transient and weak. The skin reactions of these animals 14 days after sensitization were of pure Arthus type, with no detectable delayed component. The differences between these findings and those of other workers are discussed.

in results in the appearance at the

injection site of a characteristic delayed skin reaction.

Zinsser (1921) was the first to characterize the "delayed

tuberculin type of skin reaction" as "one in which there

is no immediate effect, but in which within four, five,

or more hours, a swelling becomes apparent, which in the

course of twelve to twenty four hours reaches its

swollen condition and is characterized by the presence of

a central necrotic spot and, especially, by the presence

This reaction may not reach its highest development until

about forty-eight hours after the injection, and is

accompanied by distinct signs of inflammation and some

cell death. Delayed-type hypersensitivity is a common

response of man and experimental animals to many, if not

all, infections. It has been reported to follow infections

with other bacteria, e.g. *Brucella* (Slovak, 1956); with

various fungi (Salvin, 1963); and with viruses, e.g.

vaccinia (Smith, 1932), mumps (Enders, Cohen and Kane,

1945) and herpes simplex (Rose and Melloy, 1947). It

also accompanies the development of experimental auto-

immune diseases, such as thyroiditis (McMaster, Lerner and Evans

INTRODUCTION

When an animal (e.g., man, guinea pig or rabbit) is infected with tubercle bacilli, the intradermal injection of tuberculin results in the appearance at the injection site of a characteristic delayed skin reaction. Zinsser (1921) was the first to characterize the "delayed tuberculin type of skin reaction" as "one in which there is no immediate effect, but in which within four, five, or more hours, a swelling becomes apparent which in the course of twelve to twenty four hours results in a swollen edematous area of varying intensity, often with a central necrotic spot and, occasionally, hemorrhage. This reaction may not reach its highest development until about forty eight hours after the injection, and is accompanied by distinct signs of inflammation and some cell death". Delayed-type hypersensitivity is a common response of man and experimental animals to many, if not all, infections. It has been reported to follow infections with other bacteria, e.g. Brucella (Spink, 1956); with various fungi (Salvin, 1963); and with viruses, e.g. vaccinia (Smith, 1932), mumps (Enders, Cohen and Kane, 1945) and herpes simplex (Rose and Molloy, 1947). It also accompanies the development of experimental autoimmune diseases, such as thyroiditis (McMaster, Lerner and Exum

1961) and aspermatogenesis (Boughton and Schild, 1962) and the development of homograft immunity (Brent, Brown and Medawar, 1962).

Mycobacterium In contrast, an animal immunized against a protein in such a way as to develop circulating humoral antibody reacts to an intradermal injection of that protein with an early or Arthus-type skin reaction. Here, erythema and induration appear and reach their maximum intensity shortly after the injection and do not persist. Arthus hypersensitivity can be passively transferred to normal animals by means of specific immune serum from hypersensitive animals. Delayed-type hypersensitivity can be passively transferred to normal animals only by means of cells from sensitive animals, but not by serum (Chase, 1945).

Mycobacterium The mechanisms of delayed skin reactions could best be studied with simple, well characterized proteins instead of complex poorly characterized microbial or tissue antigens. There are several ways in which delayed type hypersensitivity to pure proteins might be induced. Dienes (1929) succeeded in inducing delayed type hypersensitivity to ovalbumin by injecting the antigen directly into tuberculous lesions of guinea pigs. Mild delayed skin reactions were reported by Jones and Mote

(1934) to occur transiently in the course of sensitization of humans by intradermal injections of foreign serum. The incorporation of antigens into adjuvants containing Mycobacteria promotes the development of delayed-type hypersensitivity to antigens such as ovalbumin but also causes increased formation of circulating antibody (Freund, 1956). More recently methods have been described for the induction of pure delayed-type hypersensitivity in the absence of circulating humoral antibody. These include the use of minute quantities of antigen (Salvin, 1958); complexes of antigen with excess antibody (Uhr, Salvin and Pappenheimer, 1957); denatured proteins (Gell and Benacerraf, 1959); and proteins coupled with haptens (Benacerraf and Gell, 1959). These methods are claimed to be effective even when Mycobacteria are not included in the sensitizing inoculum. Certain differences between hypersensitivity induced in these ways and hypersensitivity to tuberculin are, however, apparent. The "delayed-hypersensitive" state in such animals is not of long duration; the reactions appear to be very mild; and the delayed component of the skin reaction is not usually apparent except at the first skin test, after which Arthus reactions occur. Skin reactions to tuberculin, on the other hand, are frequently severe and hypersensitivity persists for a considerable

time even in the presence of circulating antibody or after repeated skin tests (Boyden and Suter, 1952).

Consideration of such differences led Raffel and Newel (1958) to group these reactions, which they termed Jones-Mote reactions, in a separate category from delayed reactions of the tuberculin type. In this paper the results are described of skin tests after sensitization of guinea pigs with small quantities of native proteins, heat denatured proteins, picrylated proteins and proteins complexed with excess antibody.

Tuberculin was obtained from the State Serum Institute, Copenhagen, Denmark. The purified protein derivative (PPD) was used for skin testing. The batches were used, RT 22 and RT 23. They were of similar potency.

Protein antigens were obtained as follows: Hen ovalbumin (OA), twice crystallized, from Nutritional Biochemicals Corporation Cleveland, Ohio and from Sigma Chemical Company, St. Louis, Missouri; Human Serum Albumin (HSA), crystallized, from Nutritional Biochemicals Corporation; Bovine Plasma Albumin (BSA) from Armour Laboratories, Eastbourne, England.

Denatured ovalbumin (DenOA) was prepared by slowly heating a solution of ovalbumin (5 mg OA per ml of distilled water) to 80°C and maintaining it at that temperature for 90 minutes (Gell and Benacerraf, 1959).

MATERIALS AND METHODS

Animals. Female albino guinea pigs weighing 400-600 grams were used. They were fed on standard pellets, supplemented with greens. Ascorbic acid was added to their drinking water.

Antigens. BCG was obtained from the Commonwealth Serum Laboratories, Melbourne, in the dried state. It was kept at 0°C and resuspended in sterile saline to a concentration of 1 mg/ml just before use.

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Picrylation of OA, HSA and BSA was carried out as described by Benacerraf and Gell (1959).

Complexes of antigen and antibody in antibody excess were prepared as follows. Rabbits were immunized against OA or HSA according to standard schedules (Boyden, 1962). The serum was separated from blood drawn from an ear vein and was stored at -20°C until use. The antibody contents of the sera were estimated by a quantitative precipitin reaction similar to that described by Gitlin (1949). To each of a series of tubes containing 0.5 ml of a solution of antigen in saline was added 0.1 ml of serum. The quantity of antigen ranged from 0 to 500 micrograms. The tubes were incubated for 1 hour at 37°C and overnight at $0-5^{\circ}\text{C}$. The precipitates which formed were washed in saline and dissolved in 4 ml 0.1 N NaOH. The optical density of these solutions was measured at a wavelength of 2800 Å in a Beckman DU spectrophotometer. The precipitin curve was plotted and from this were determined the antibody content and the maximum antigen-combining capacity of the serum. Precipitates were prepared by adding to the appropriate quantity of serum half the maximum quantity of antigen which could be bound. The mixture was incubated at 37°C for 2 hours and overnight at $0-5^{\circ}\text{C}$. The precipitates were washed and resuspended in saline to the concentration required for sensitization

of guinea pigs. The maximum antigen-binding capacities of the sera used were: anti-OA, 150 micrograms per ml serum; anti-HSA, 625 micrograms per ml serum.

Sensitization of animals. Saline solutions or suspensions of the antigens were emulsified with equal volumes of either Freund's Incomplete Adjuvant (Difco) or Freund's Complete Adjuvant (Difco), the latter containing killed Mycobacterium butyricum. Emulsions in incomplete adjuvant are referred to as antigen in oil. An injection of 0.25 ml was given into each hind foot pad, 5 micrograms of antigen being included in the total volume of 0.5 ml. Animals were sensitized to tuberculin by a single intradermal injection of BCG (0.1 mg) 4 weeks before skin testing.

Skin testing. Routinely, animals were skin tested 7 days and/or 14 days after sensitization. The skin of the flanks was shaved 3 hours before the test. Five or ten micrograms of antigen in 0.1 ml saline were injected intradermally. The thickness of a fold of skin at the site of injection was measured by means of skin calipers (Schnelltaster, System Kröplin Type A. 02 T, H.C. Kröplin GmbH, Schlüchtern, Hessen, Germany). Measurements were made immediately before injection and 3, 6, 12, 24, 30, 48 and sometimes 72 hours afterwards. The results are

expressed below as "specific increase in skin thickness".

This represents the increase in skin thickness (in mm)

in sensitive animals, less the mean increase in skin thickness in normal animals tested at the same time.

In addition, the diameter of the erythema at the site

was measured and the degree of erythema arbitrarily

graded, from \pm to +++.

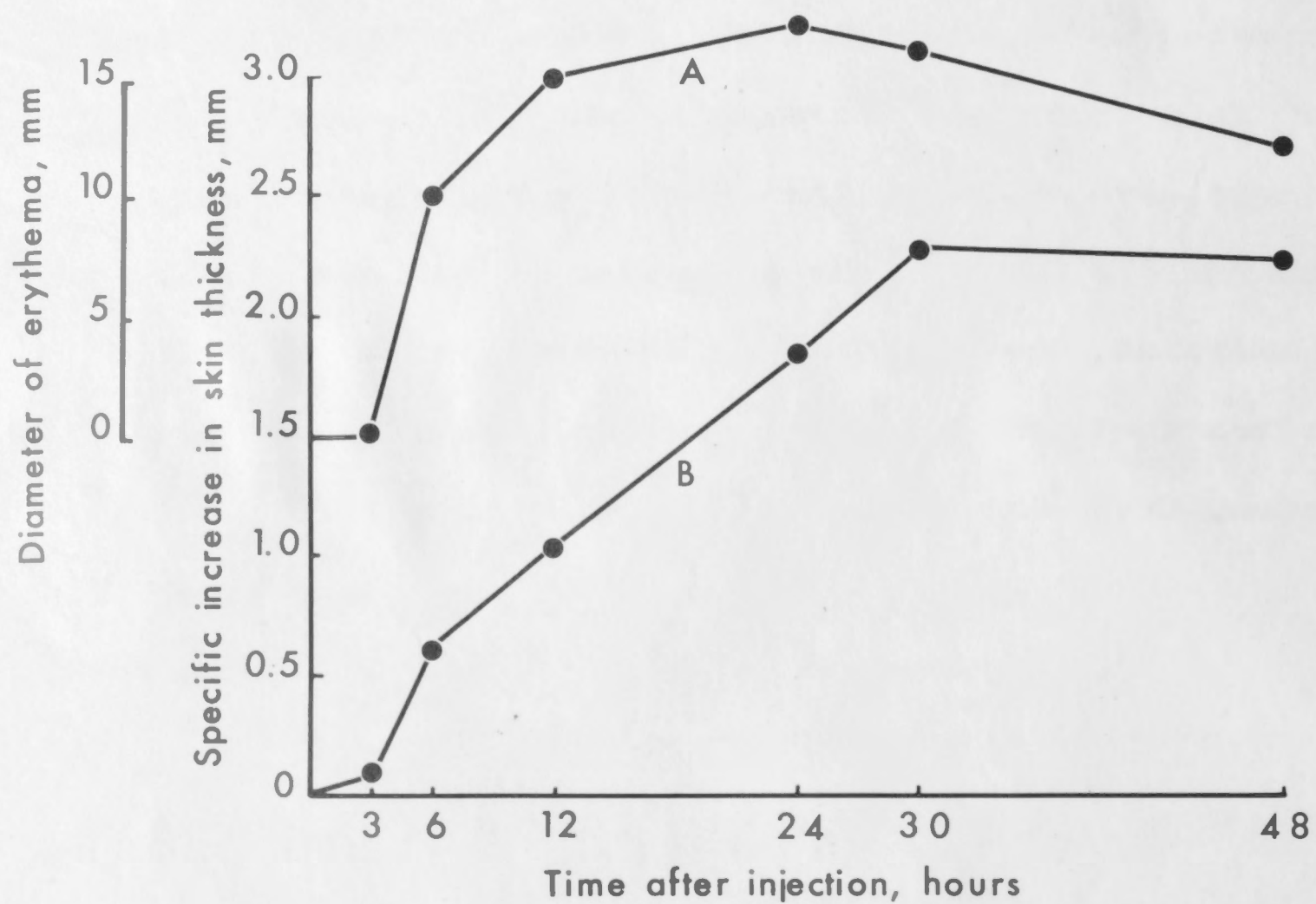


Figure 1. Skin reactions to PPD in BCG-vaccinated guinea pigs.

A: Mean diameter of erythema.

B: Mean specific increase in skin thickness.

RESULTS

TUBERCULIN SENSITIVITY

Female guinea pigs were injected intradermally with 10 micrograms PPD 4 weeks after immunization with 0.1 mg BCG. Figure 1 shows the results of the skin tests expressed as mean specific increase in skin thickness and mean diameter of erythema. These results illustrate the features of the delayed hypersensitivity resulting from infection with tubercle bacilli - the classical tuberculin reaction. The features are the virtual absence of a reaction at 3 hours, with a very slight reaction at 6 hours, increasing steadily to reach a peak at 24-30 hours and persisting for 48 to 72 hours. The area and degree of erythema increase approximately in parallel with the degree of skin thickening, though erythema is maximal rather earlier.

SENSITIZATION WITH NATIVE PROTEINS

Groups of guinea pigs were injected in the hind foot pads with 5 micrograms of OA, HSA or BSA emulsified in either oil or complete adjuvant. They were skin tested with 5 micrograms of the same protein 7 and 14 days later.

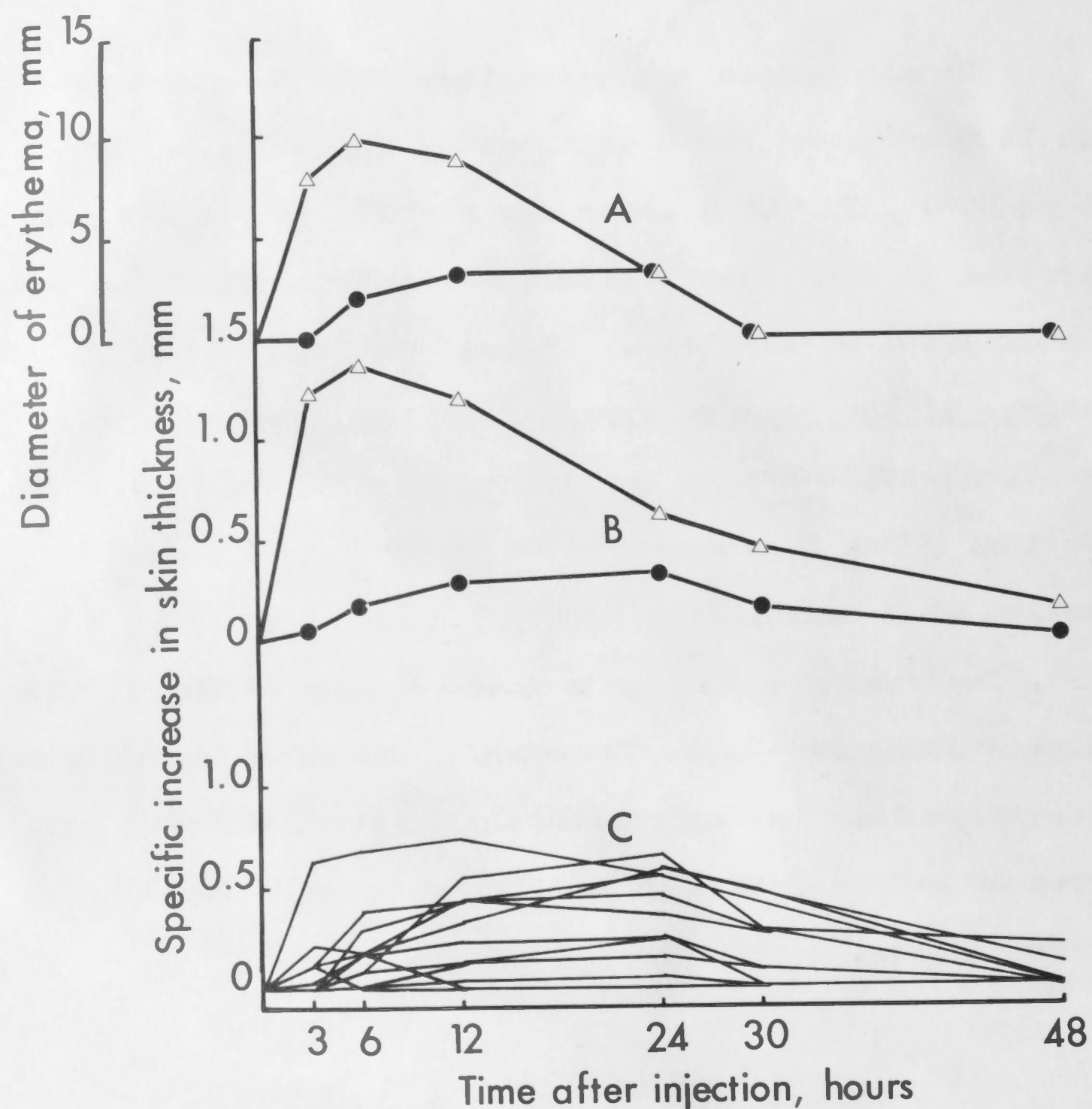


Figure 2. Skin reactions to OA in guinea pigs sensitized with OA in oil. ●-● 1st test 7 days after sensitization. Δ-Δ, 1st test 14 days after sensitization. A: Mean diameter of erythema. B: Mean specific increase in skin thickness. C: Individual reactions at 7 days.

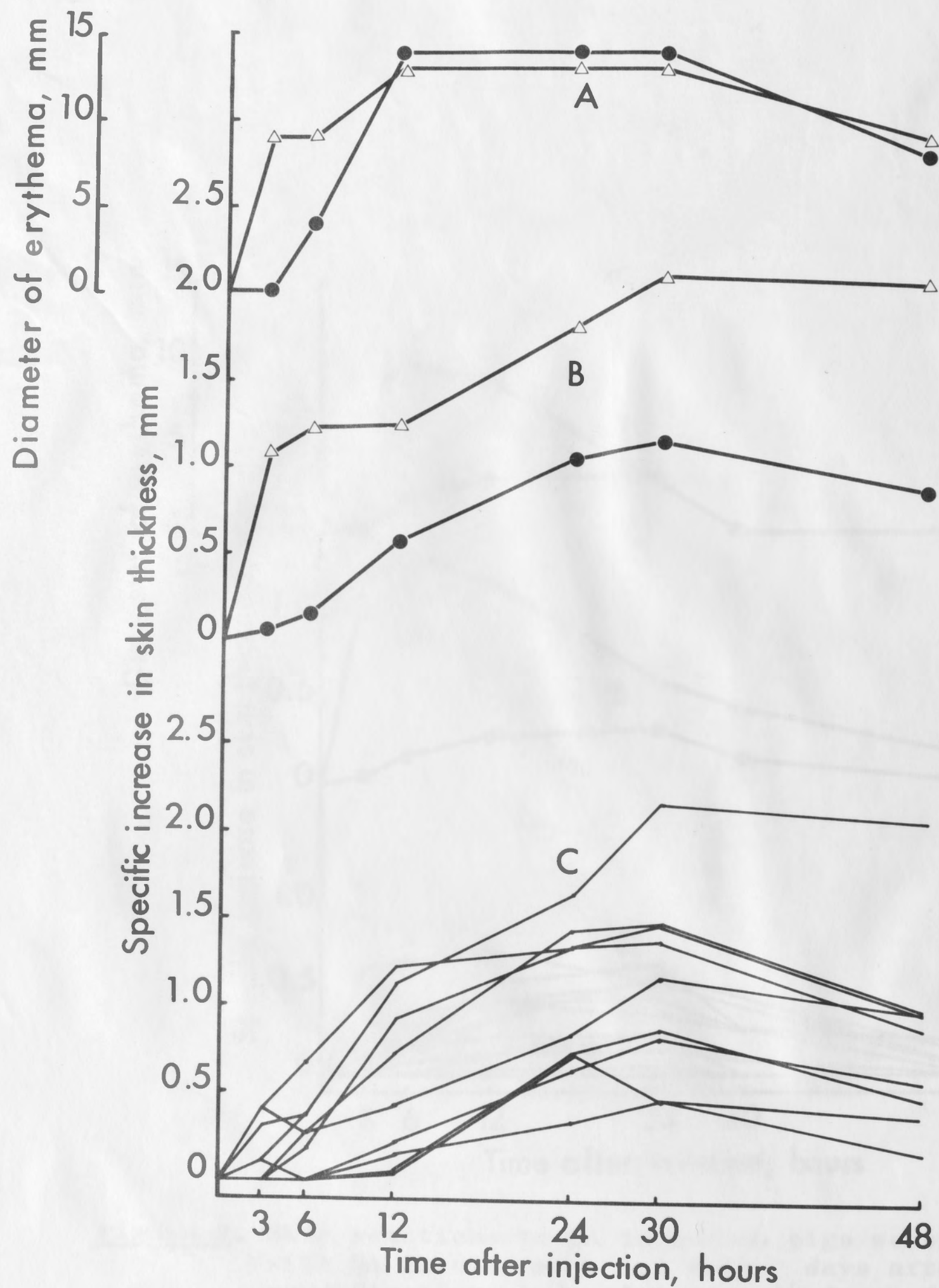


Figure 3. Skin reactions to OA in guinea pigs sensitized with OA in complete adjuvant. Symbols as in Figure 2.

There were 6-10 animals in each group tested. The results of the skin tests are shown graphically in Figures 2, 3, 4 and 5.

Ovalbumin. Animals sensitized with OA in oil (Figure 2) showed weak responses to intradermal testing 7 days after sensitization. The mean increase in skin thickness was maximal at 24 hours and declined thereafter, there being no reaction at 48 hours. Erythema was maximal at 12 to 24 hours but had disappeared at 30 hours. The skin thickening in individual animals (Figure 2C) reached a maximum at any time from 3 to 24 hours. Fourteen days after sensitization these animals were re-tested and another group was tested for the first time. Both these groups reacted similarly, but only the reactions of the latter group are shown in the Figure. These reactions were maximal at 3 to 6 hours and declined steadily thereafter. They were still detectable, though declining, 24 hours after the skin test.

A different pattern of reactivity to skin tests was apparent in the animals sensitized with OA in complete adjuvant (Figure 3). In animals tested 7 days after sensitization the reactions at 3 and 6 hours were small or absent. Skin thickening was maximal at 30 hours but was fairly constant from 24 to 48 hours. Erythema was equally marked from 12 to 30 hours. Though there was considerable

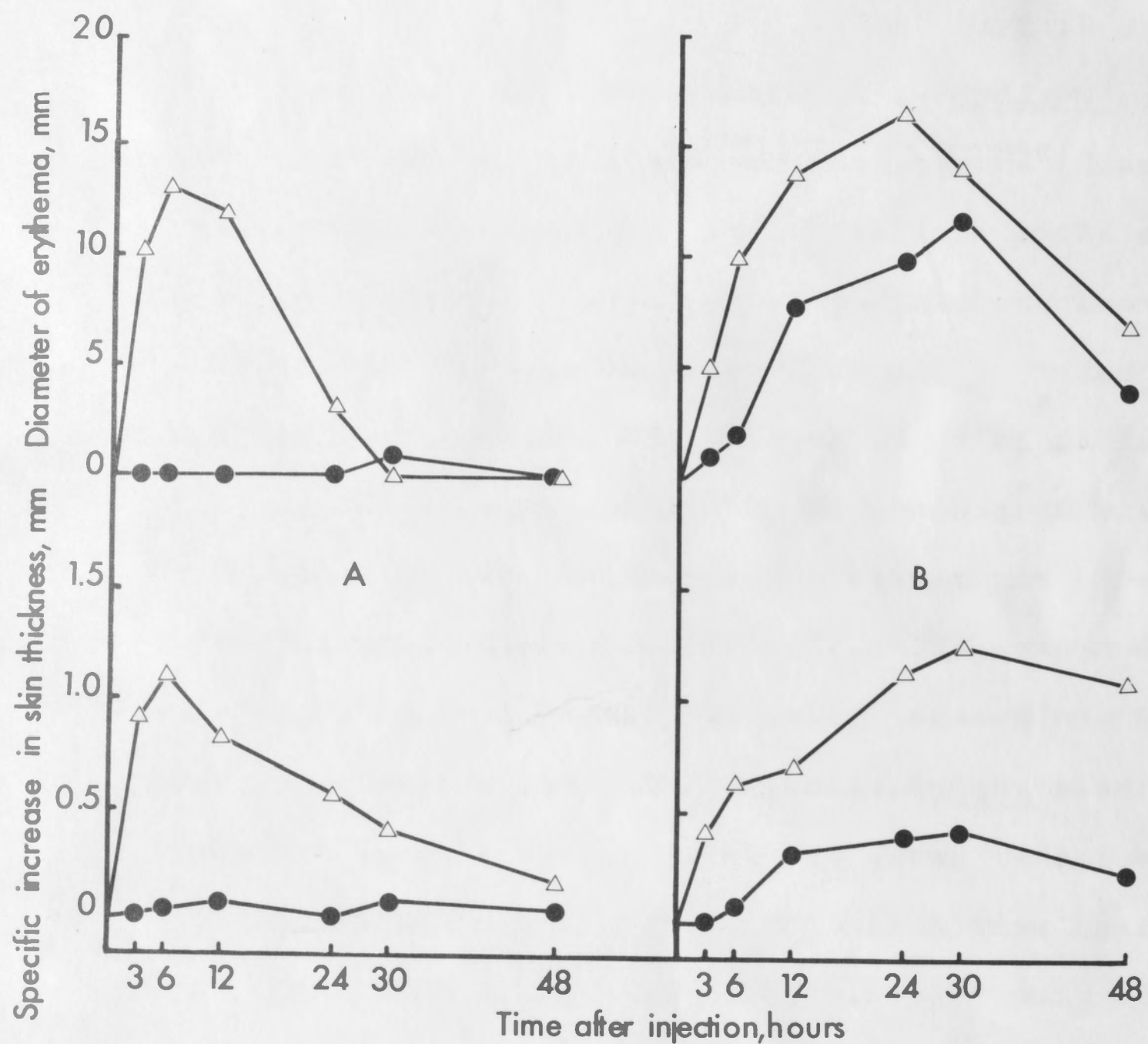


Figure 4.

Mean skin reactions to HSA in guinea pigs sensitized with HSA in oil (A) or complete adjuvant (B) and tested 7 days (●—●) or 14 days (Δ—Δ) after sensitization.

variation in degree, the pattern of increases in skin thickness with time was similar in different animals (Figure 3C). In animals re-tested or tested for the first time 14 days after sensitization erythema and induration were both quite marked 3 and 6 hours after skin testing. Between 6 and 30 hours there was a further progressive increase in skin thickness, which had decreased only slightly 48 hours and 72 hours after injection. The diameter of the area of erythema decreased rather more quickly.

Human Serum Albumin (Figure 4). In animals skin tested 7 days after sensitization with HSA in oil there was practically no response. In animals tested for the first time or re-tested 14 days after sensitization the reactions were maximal 6 hours after injection. Erythema and induration became progressively less intense thereafter. The reactions to HSA in these animals were less intense than those to OA in animals sensitized with OA in oil.

A different pattern of reactivity was seen in animals skin tested after sensitization with HSA in complete adjuvant. Those tested 7 days after sensitization had no reactions at 3 hours, and slight reactions at 6 hours after which erythema and induration increased up to 30 hours and were still present at 48 hours. Those tested

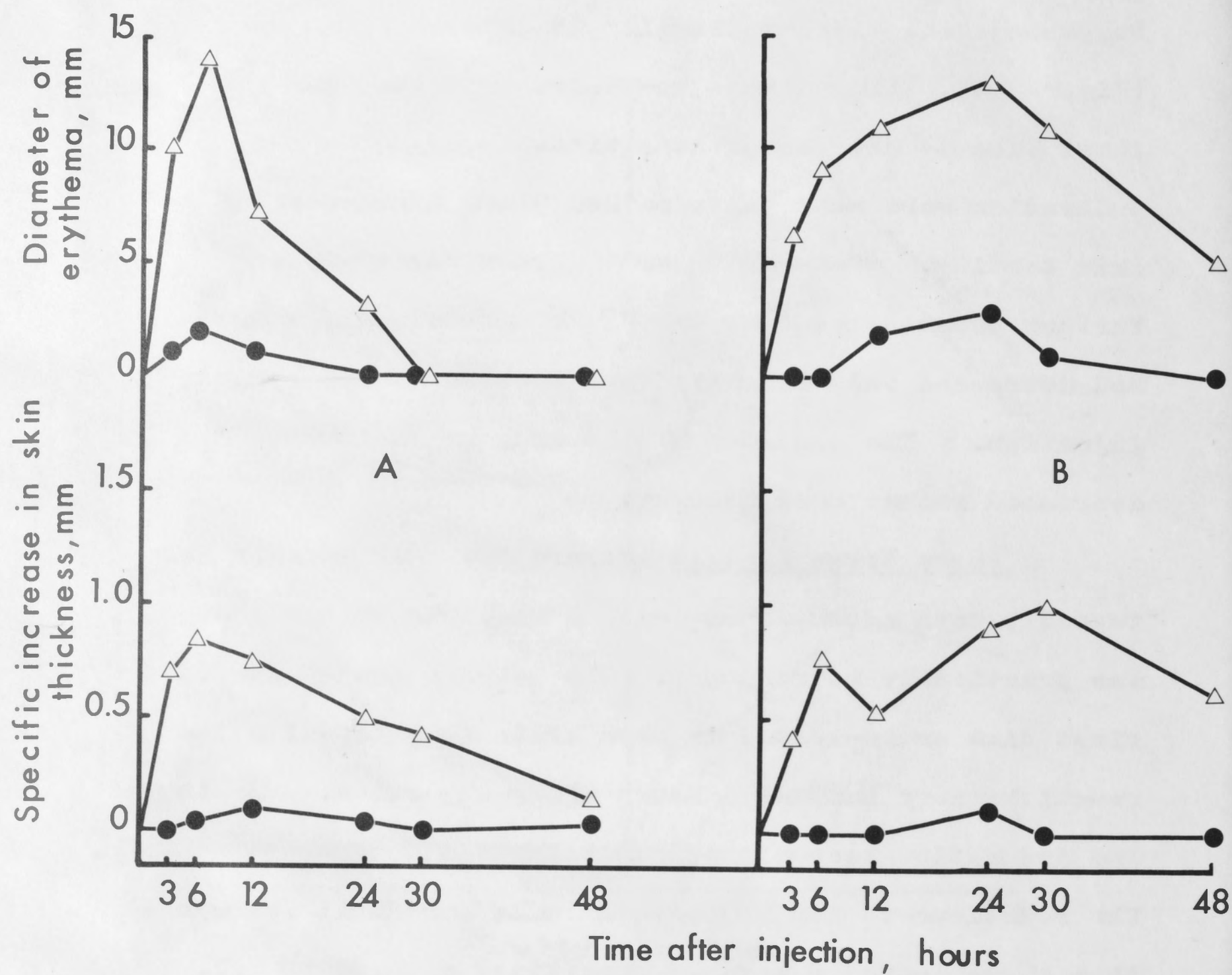


Figure 5. Mean skin reactions to BSA in guinea pigs sensitized with BSA in oil (A) or complete adjuvant (B) and tested 7 days (● — ●) or 14 days (Δ — Δ) after sensitization.

14 days after sensitization had mild early reactions which gave way to more marked erythema and induration. These were maximal at 24 to 30 hours and still present at 48 hours.

Bovine Serum Albumin (Figure 5). Animals sensitized with BSA in oil showed little or no reaction to skin tests 7 days after sensitization and early reactions 14 days after sensitization. In animals skin tested 7 days after sensitization with BSA in complete adjuvant there was a small increase in skin thickness at 24 hours and slight erythema maximal at 24 hours. In animals tested 14 days after sensitization with BSA in complete adjuvant there was a rapid increase in skin thickness up to 6 hours, a slight decrease, then a further increase to a maximum at 30 hours. Skin thickening was still marked at 48 hours. Erythema was maximal at 24 hours and still present at 48 hours.

SENSITIZATION WITH DENATURED OVALBUMIN

Guinea pigs were injected in the hind foot pads with 5 micrograms heat denatured OA (Den-OA) either in oil or in complete adjuvant. Four guinea pigs in each group were skin tested with 5 micrograms native ovalbumin 7 or 14 days later.

In animals sensitized with Den-OA in oil there were no reactions to skin tests 7 days after sensitization, but fairly intense early reactions 14 days after sensitization. Those sensitized with Den-OA in complete

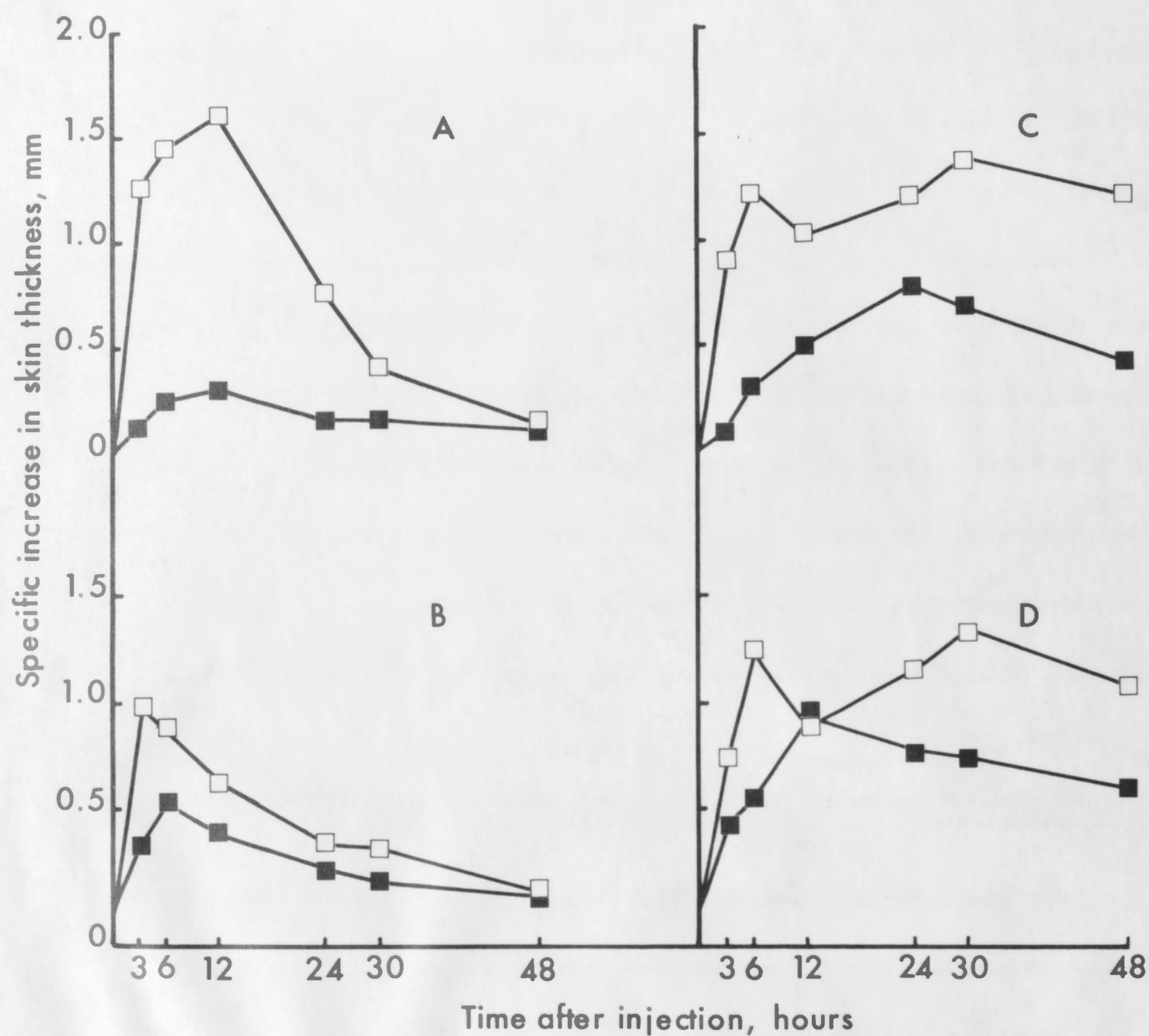


Figure 6. Skin reactions (mean specific increases in skin thickness) in guinea pigs sensitized with Pic-OA in oil (A and B) or complete adjuvant (C and D) and tested 7 days (■ — ■) or 14 days (□ — □) after sensitization. Upper curves (A and C), tested with OA; lower curves (B and D), tested with Pic-OA.

adjuvant showed marked delayed reactions similar to those obtained after sensitization with native proteins in complete adjuvant. These delayed reactions were accompanied by Arthus reactions only in the animals tested 14 days after sensitization.

SENSITIZATION WITH PICRYLATED PROTEINS

Guinea pigs were injected in the hind foot pads with 5 micrograms picrylated ovalbumin (Pic-OA), picrylated HSA (Pic-HSA) or picrylated BSA (Pic-BSA). Groups of 6 or 8 animals were skin tested 7 or 14 days later with both the native protein and the picrylated protein. The results of these tests, expressed as mean specific increase in skin thickness, are shown in Figures 6, 7 and 8.

Picrylated ovalbumin (Figure 5). In animals tested 7 days after sensitization with Pic-OA in oil the reactions to Pic-OA were slight but were maximal at 6 hours. The reactions to OA were also slight but were maximal at 12 hours. In animals tested 14 days after sensitization the reactions to Pic-OA were maximal at 6 hours and had disappeared by 24 hours. The reactions to OA were stronger but still maximal at 12 hours, after which they declined rapidly and had virtually disappeared by 48 hours.

Again, different reactions were seen when animals

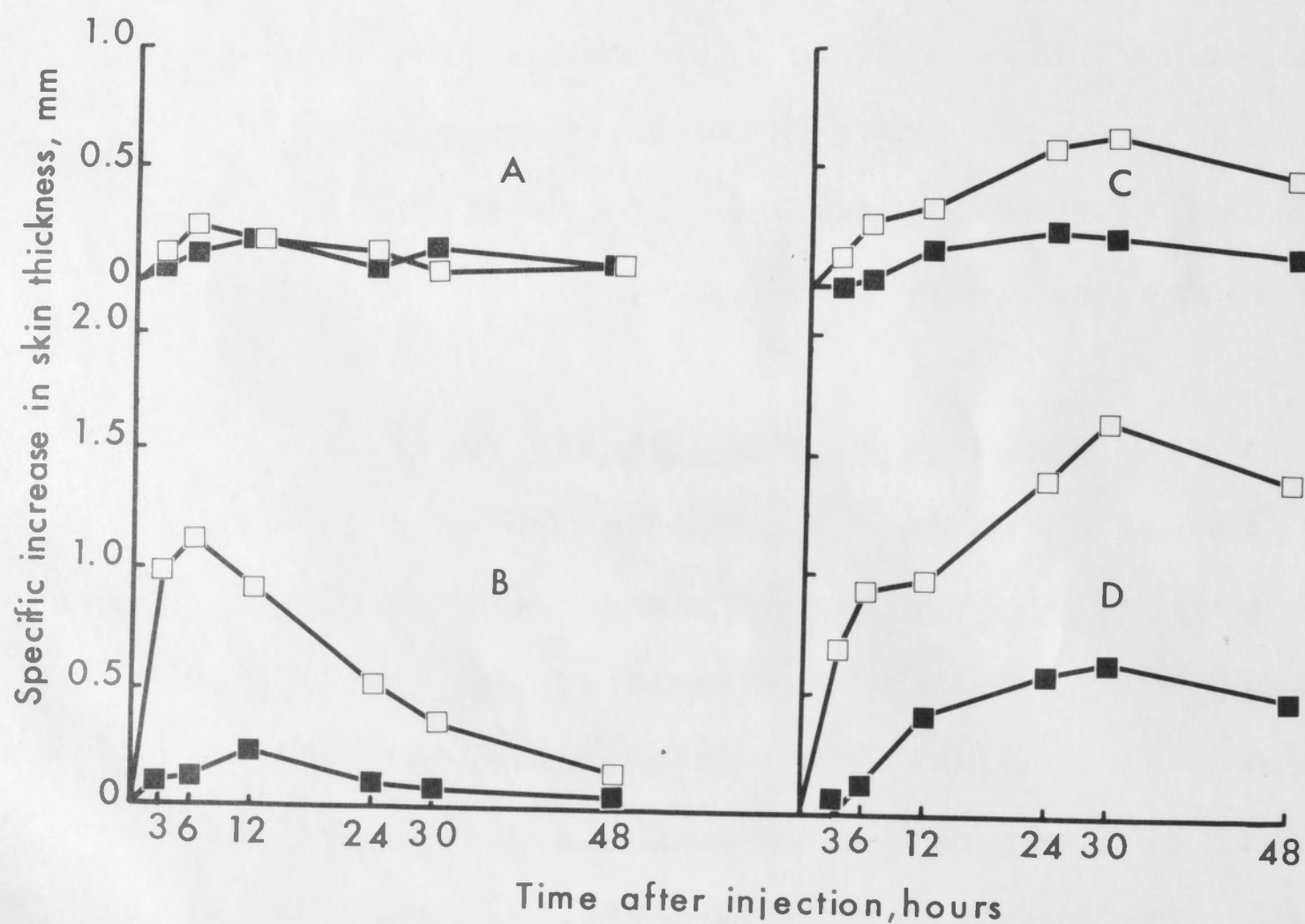


Figure 7. Skin reactions (mean specific increases in skin thickness) in guinea pigs sensitized with Pic-HSA in oil (A and B) or complete adjuvant (C and D) and tested 7 days (■—■) or 14 days (□—□) after sensitization. Upper curves (A and C), tested with HSA, lower curves (B and D) tested with Pic-HSA.

sensitized with Pic-OA in complete adjuvant were skin tested with either OA or Pic-OA. The reactions at 3 and 6 hours were slight in the animals tested 7 days after sensitization but quite marked in those tested 7 days later. Delayed reactions occurred which were maximal between 12 and 30 hours and persisted to 48 hours. The delayed reactions were more intense in the animals tested 14 days after sensitization.

Picrylated Human Serum Albumin (Figure 7).

Animals tested 7 days after sensitization with Pic-HSA in oil reacted very weakly to Pic-HSA, these reactions being maximal at 12 hours. The reactions to skin tests with Pic-HSA 14 days after sensitization were maximal at 6 hours and then declined quickly. At both times reactions to HSA were barely detectable.

Animals sensitized with Pic-HSA in complete adjuvant had delayed skin reactions to Pic-HSA 7 days after sensitization, skin thickening being maximal at 30 hours and still marked at 48 hours. Fourteen days after sensitization these reactions were stronger and accompanied by reactions at 3 and 6 hours. The reactions to HSA at both times were much weaker, though delayed. Skin thickening was slight at 3 to 6 hours, maximal at 24 to 30 hours and still present at 48 hours.

Picrylated Bovine Serum Albumin (Figure 8). The

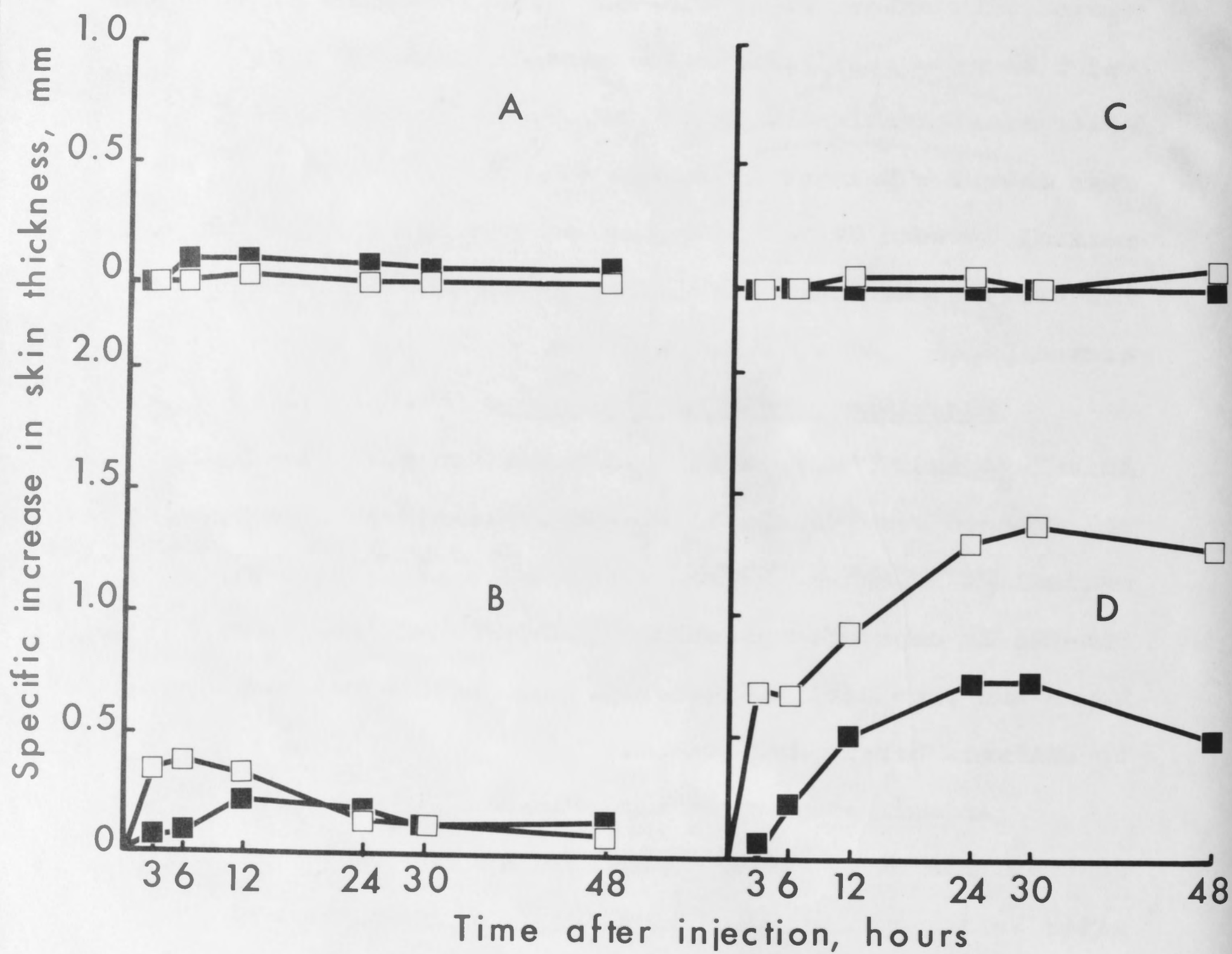


Figure 8. Skin reactions (mean specific increases in skin thickness) in guinea pigs sensitized with Pic-BSA in oil (A and B) or complete adjuvant (C and D) and tested 7 days (■—■) or 14 days (□—□) after sensitization. Upper curves (A and C), tested with BSA; lower curves (B and D), tested with Pic-BSA.

reactions to unconjugated BSA were barely detectable in any of the animals sensitized with Pic-BSA either in oil or in complete adjuvant. In animals sensitized with Pic-BSA in oil, the reactions to Pic-BSA 7 days after sensitization consisted only of slight transient thickening and erythema at 24 hours. Those tested with Pic-BSA 14 days after sensitization showed weak reactions at 3 and 6 hours.

In animals tested with Pic-BSA 7 days after sensitization with Pic-BSA in complete adjuvant the reactions were slight at 3 to 6 hours, maximal at 24 to 30 hours and still present at 48 hours. In those tested with Pic-BSA 14 days after sensitization there were reactions at 3 and 6 hours and more marked reactions at 24 to 30 hours, persisting to 48 hours.

SENSITIZATION WITH ANTIGEN-ANTIBODY COMPLEXES

Two series of guinea pigs were immunized with 5 micrograms OA or HSA in the form of complexes with excess antibody. Group A (12 animals in each series) received the complex emulsified in incomplete adjuvant. Group B (12 animals in each series) received the complex in complete adjuvant. Half the animals in each group were skin tested 7 and 14 days after sensitization; half were skin tested only 14 days after sensitization.

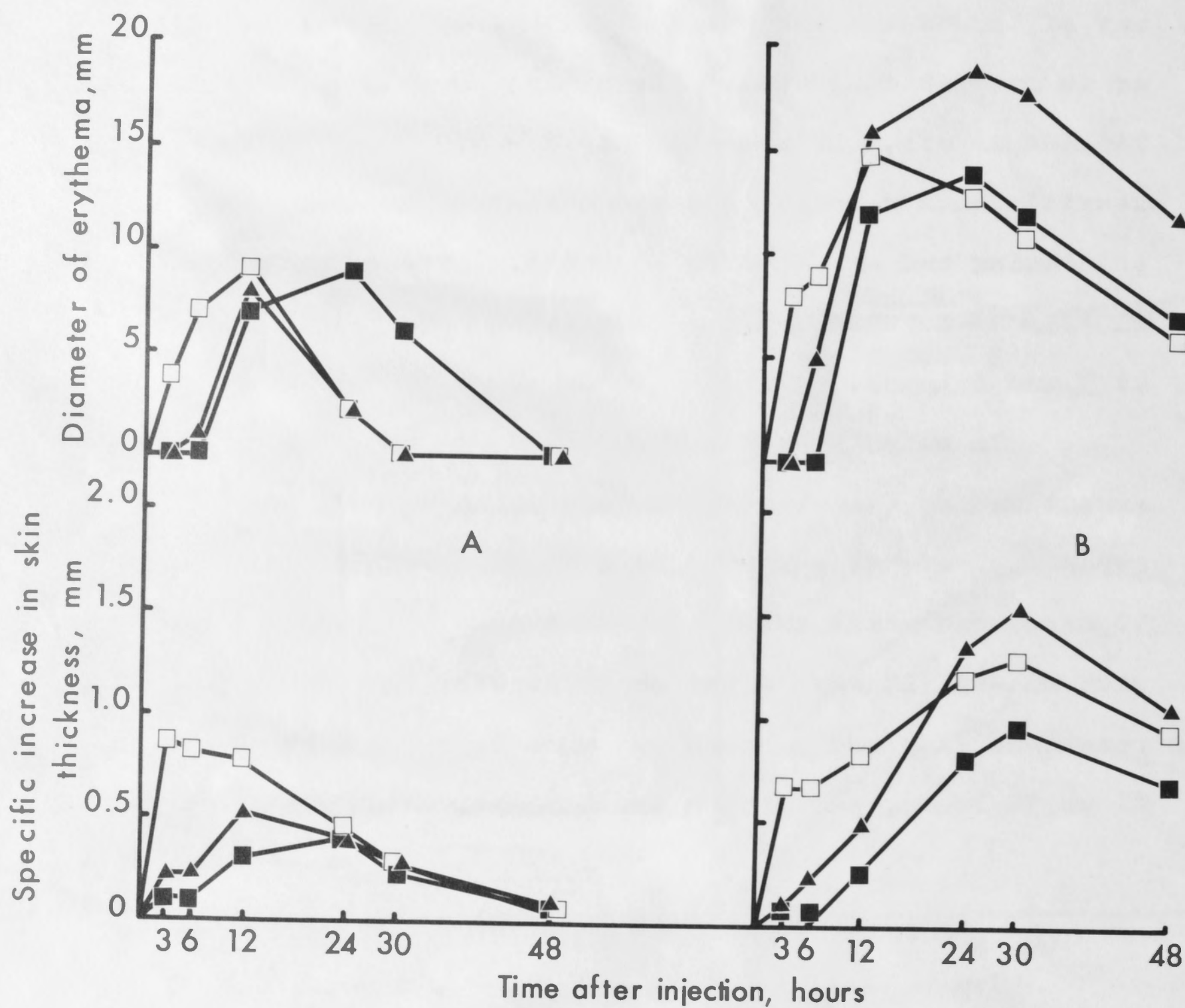


Figure 9. Mean skin reactions to OA in guinea pigs sensitized with OA-anti OA complexes in oil (A) or complete adjuvant (B). Reactions to first test 7 days (■ — ■) or 14 days (▲ — ▲) after sensitization; and to second test 14 days (□ — □) after sensitization.

Ovalbumin. (Figure 9) shows the results of the skin tests, expressed as mean specific increase in skin thickness and mean diameter of erythema. In animals tested for the first time 7 days after sensitization with complex in oil there was slight skin thickening which was delayed in onset, maximal at 24 hours and had disappeared by 48 hours. This was accompanied by erythema which, though slight in degree, was of considerable diameter. When these animals were retested 7 days later, the reactions were maximal at 6 to 12 hours and then declined. The animals in Group A tested for the first time 14 days after sensitization showed slight skin thickening and erythema maximal at 12 hours.

The animals of Group B tested 7 days after sensitization showed pure delayed reactions, maximal at 30 hours and persisting beyond 48 hours. Virtually pure delayed reactions were also seen in the animals tested for the first time 14 days after sensitization. The animals retested at this time had mild early reactions as well as delayed reactions.

Human Serum Albumin (Figure 10). Animals of Group A tested 7 days after sensitization showed mild reactions, with slight increases in skin thickness at 12 to 24 hours, disappearing by 48 hours. The same animals retested 7 days later showed reactions which were maximal

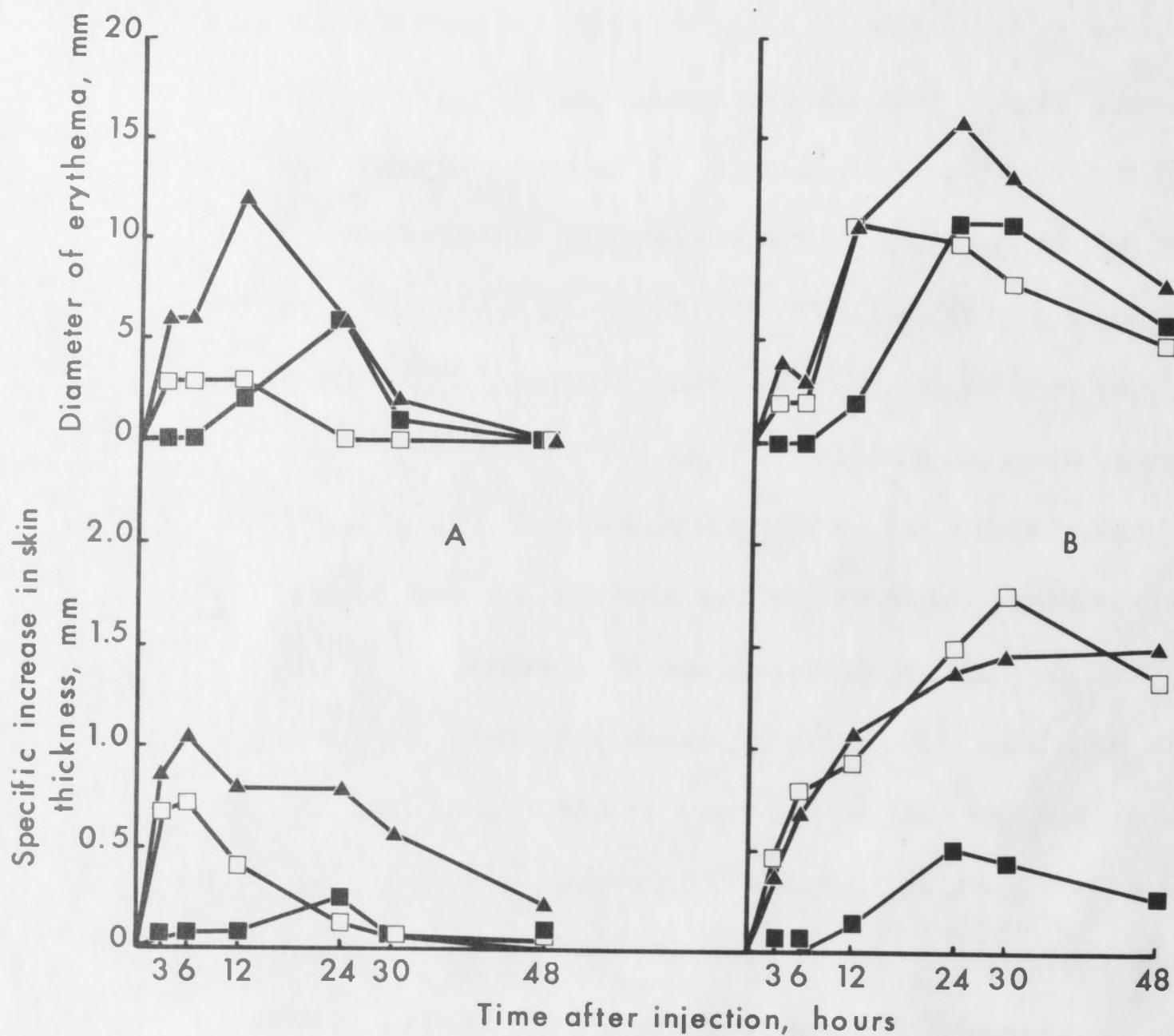


Figure 10. Mean skin reactions to HSA in guinea pigs sensitized with HSA-anti HSA complexes in oil (A) or complete adjuvant (B). Reactions to first test 7 days (■—■) or 14 days (▲—▲) after sensitization; and to second test 14 days (□—□) after sensitization.

at 3 to 6 hours and declined thereafter. Animals tested for the first time 14 days after sensitization showed reactions maximal at 6 to 12 hours and declining slowly thereafter.

Animals of Group B showed delayed reactions when skin tested 7 or 14 days after sensitization. These reactions were very weak and unaccompanied by an Arthus component in those tested 7 days after sensitization, but stronger and accompanied by mild Arthus reactions in those tested or retested 7 days later.

SENSITIZING EFFECT OF DIFFERENT DOSES OF ANTIGEN

Salvin (1958) found that the duration of the "delayed hypersensitivity" following sensitization with small amounts of pure proteins was to some extent inversely proportional to the dose of antigen used. We have investigated the possibility that this might account for the discrepancy between his findings and ours and more particularly for our failure to demonstrate delayed reactions to HSA after sensitization with antigen in oil.

Groups of guinea pigs (4 or more to a group) were sensitized with HSA in oil or in complete adjuvant. The dose of HSA ranged from 0.05 to 50 micrograms. The animals were skin tested 14 days later with 5 micrograms HSA. The results, expressed as mean specific increases

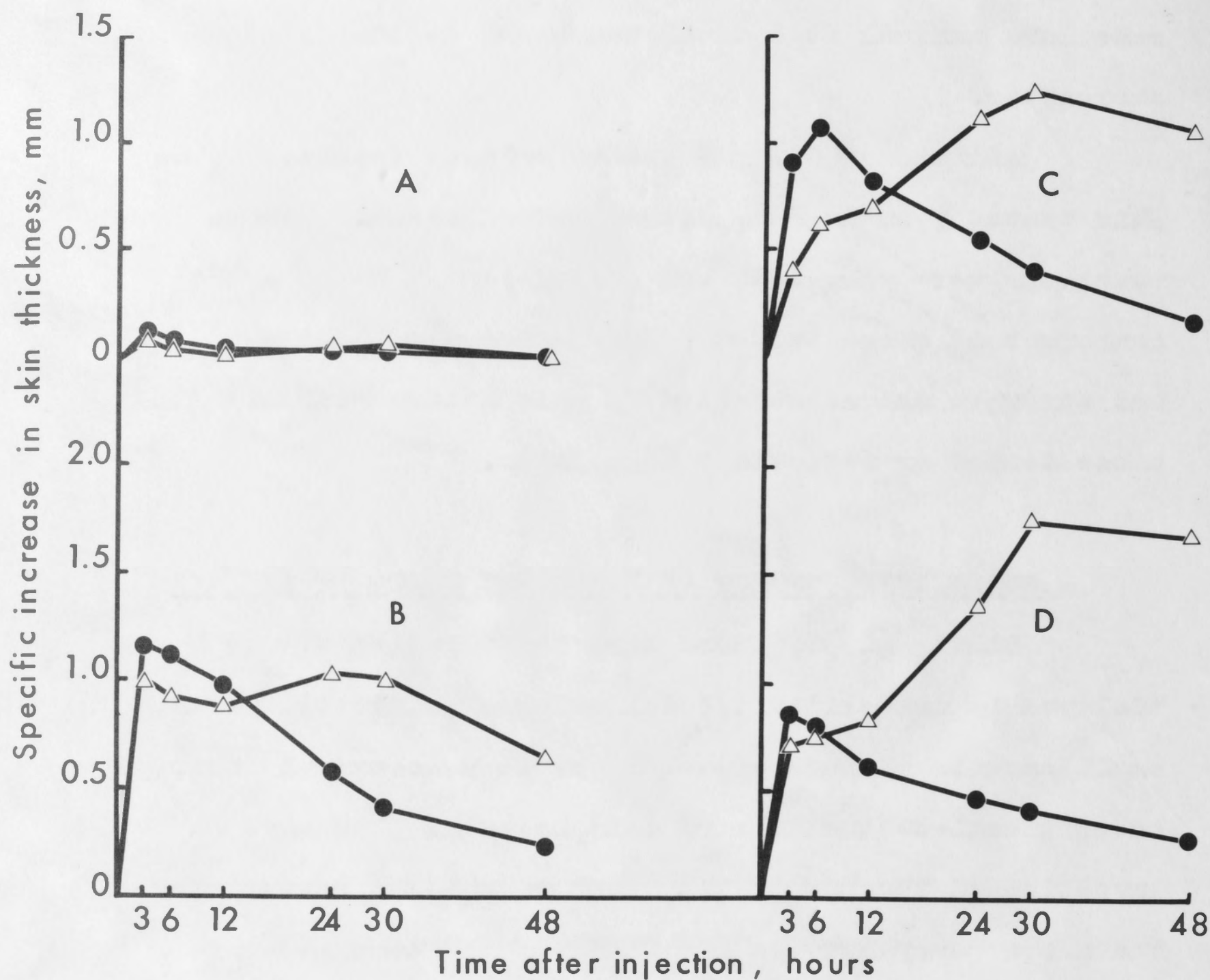


Figure 11. Skin reactions (mean specific increases in skin thickness) to HSA in guinea pigs sensitized 14 days previously with different doses of HSA in oil (● — ●) or complete adjuvant (Δ — Δ). A, 0.05 micrograms; B, 0.5 micrograms; C, 5 micrograms; D, 50 micrograms.

in skin thickness at the times shown, are presented in Figure 11. The animals sensitized with 0.05 micrograms HSA in oil or complete adjuvant hardly reacted at all. It can be seen that in the other groups early reactions without delayed reactions to HSA developed in animals sensitized with HSA in oil. The animals sensitized with HSA in complete adjuvant all had early reactions (3 to 6 hours) with marked delayed reactions (24 to 48 hours).

CROSS REACTIONS AFTER SENSITIZATION WITH PICRYLATED PROTEINS

Six guinea pigs were sensitized with 5 micrograms Pic-HSA in complete adjuvant. Two weeks later they were all skin tested with 5 micrograms each of Pic-HSA, Pic-BSA, Pic-OA, HSA, BSA and OA. The results, expressed as mean specific increase in skin thickness, are shown in Figure 12. Early reactions (3 to 6 hours) occurred in response to the hapten, no matter to which protein it was conjugated. These were associated with delayed reactions maximal at 24 to 30 hours and persisting to 48 hours in response to Pic-HSA and Pic-BSA. There were weak delayed reactions to HSA and no significant reactions to BSA or OA. There thus appeared to be some specificity associated with the hapten-protein conjugate as well as with the protein itself.

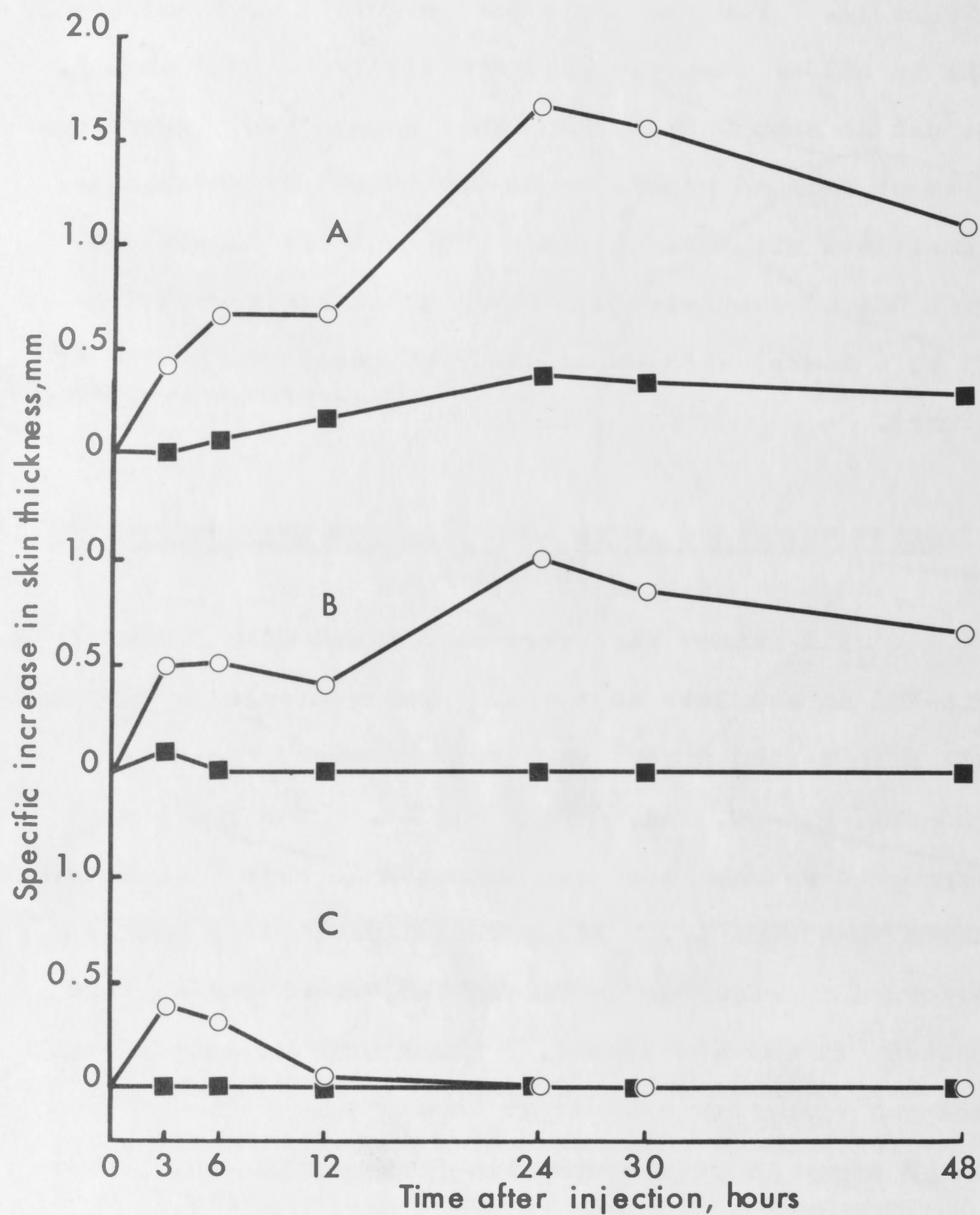


Figure 12. Skin reactions (mean specific increase in skin thickness) to native (■—■) and picrylated (○—○) proteins in guinea pigs sensitized with Pic-HSA in complete adjuvant. A, HSA and Pic-HSA; B, Pic-BSA and BSA; C, Pic-OA and OA.

In another experiment it was noted that animals with strong early and delayed reactions to Pic-BSA following sensitization with Pic-BSA in complete adjuvant showed pure Arthus reactions when tested with Pic-OA.

EFFECT OF PREVIOUS SKIN TESTS ON REACTIONS TO HSA

It is known that repeated skin testing does not abolish delayed cutaneous reactivity in tuberculin-sensitive animals. The "delayed" reactions seen after sensitization with pure protein antigens in oil are usually not seen at any but the first skin test. The effect of repeated testing on the delayed reactions seen after sensitization with antigen in complete adjuvant was also investigated.

Four groups of guinea pigs were sensitized with 5 micrograms HSA in complete adjuvant. They were skin tested with 5 micrograms HSA at the following times after sensitization: Group A (6 animals), 7, 14, 21 and 28 days; Group B (6 animals), 14, 21 and 28 days; Group C (6 animals), 21 and 28 days; Group D (7 animals), 28 days. The results of the tests, expressed as mean specific increase in skin thickness, are shown in Figure 13.

It can be seen that in Groups A and B delayed reactions to skin tests became progressively more intense

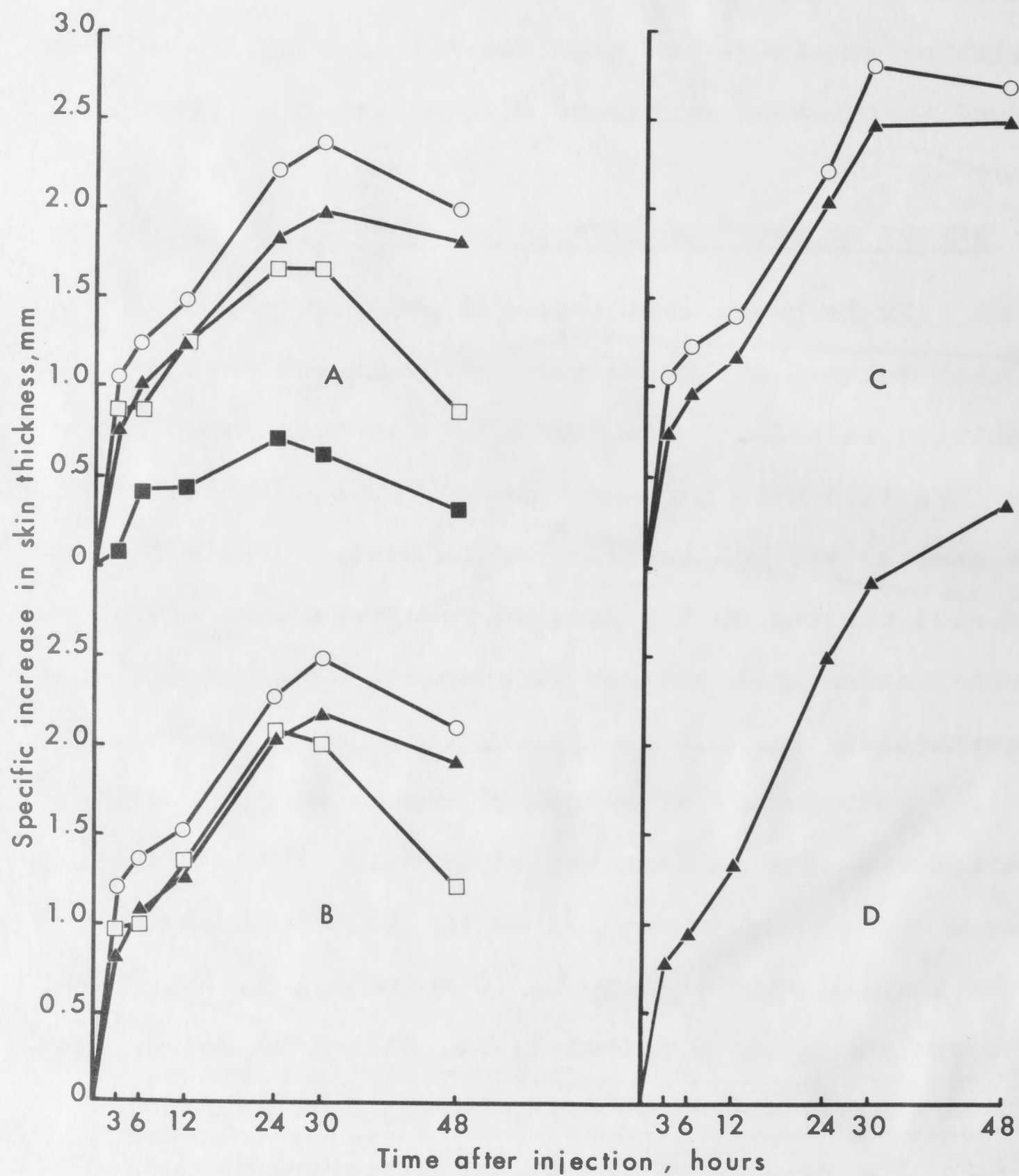


Figure 13. Skin reactions to HSA in guinea pigs sensitized with HSA in complete adjuvant and tested 4 times (A), 3 times (B), twice (C) or once (D). Mean specific increases in skin thickness at tests 7 days (■-■), 14 days (□-□), 21 days (○-○) or 28 days (▲-▲) after sensitization.

up to 21 days and were much the same 28 days after sensitization. In Group C the reactions 21 and 28 days after sensitization were similar to each other and to the reactions at these times in Groups A and B. The reactions in Group D were slightly more intense and prolonged than in any of the other Groups.

Thus repeated skin testing is not associated with a progressive decrease in the delayed cutaneous reactivity although it may slightly decrease the reactivity in comparison with animals not previously skin tested.

2. Delayed skin reactions similar to those observed in BCG vaccinated animals skin tested with tuberculin (PPD). These are characterized, in their pure form, by a delayed onset of erythema and induration which first appear after 6 hours,

DISCUSSION

In these experiments the reactions to skin tests following sensitization of guinea pigs with small amounts of native proteins or proteins modified in different ways have been observed. They can be grouped in four categories:

1. Reactions characterized by erythema and skin thickening which become apparent shortly after intradermal injection of the antigen, are maximal at 6 hours or occasionally at 12 hours, decline rapidly and disappear completely between 24 and 48 hours. Such reactions were seen only in animals sensitized with antigen in oil and were usually most intense in those tested 14 days after sensitization. Although they differ from the reactions in rabbits originally described by Arthus (1903), we have followed the present custom and called these Arthus reactions.
2. Delayed skin reactions similar to those observed in BCG vaccinated animals skin tested with tuberculin (PPD). These are characterized, in their pure form, by a delayed onset of erythema and induration which first appear after 6 hours,

reach their maximum intensity after about 24 to 30 hours and are still fairly marked after 48 to 72 hours. In animals sensitized with native or modified protein antigens such delayed reactions were seen only when the sensitizing injection was incorporated in Freund's complete adjuvant (containing Mycobacteria). They were seen in nearly all such animals tested 7 days after sensitization but also occurred in animals tested 14 days after sensitization with antigen-antibody. We consider such reactions to be those of classical delayed-type hypersensitivity.

3. Reactions present at 3 to 6 hours, often decreasing in size and later increasing again to become maximal at 24 to 30 hours, and persisting to 48 hours. These were seen in most animals tested for the first time or retested 14 days after sensitization with native or modified proteins in complete adjuvant. We consider that these represent a mixture of Arthus and delayed reactions.
4. Reactions which were characterized by slight thickening of the skin accompanied by erythema

which was of slight intensity but often covered quite a large area. The onset of these reactions was delayed (12 to 30 hours) but they did not persist. These reactions occurred in animals tested 7 days after sensitization with antigen in incomplete adjuvant. They occurred: after sensitization with OA (but not HSA or BSA); or after sensitization with antigen-antibody complexes; in response to Pic-HSA and HSA in animals sensitized with Pic-HSA; and in response to Pic-BSA in animals sensitized with Pic-BSA. Not only were the reactions transient, but the state of hypersensitivity in which animals reacted in this way was also transient, as none of the animals in this series of experiments reacted in this way when tested 14 days after sensitization. In contrast, the delayed reactions occurring in animals sensitized with antigen in complete adjuvant became stronger as the time interval between sensitization and skin test increased, with or without repeated skin tests. We have called this fourth type of reaction the Jones-Mote reaction, following the usage of Raffel and Newel (1958).

These findings appear to be different from those of other workers. One possible explanation of these differences may lie in the use of different methods of reading the skin reactions. Salvin (1958) injected guinea pigs with small quantities of diphtheria toxoid or ovalbumin in incomplete adjuvant (lacking Mycobacteria) and observed a phase of "delayed hypersensitivity" preceding Arthus hypersensitivity. In these experiments, skin reactions were examined 4 and 24 hours after skin testing, at which times the area of induration was noted. Neither the degree of induration nor observations at other times were reported. Benacerraf and Gell (1959) and Gell and Benacerraf (1959), studying the skin reactions in guinea pigs sensitized with protein conjugates or denatured proteins in incomplete adjuvant, recorded no observations beyond 24 hours. The methods used by Uhr, Salvin and Pappenheimer (1957) and Sell and Weigle (1959) to record the skin reactions of guinea pigs sensitized with antigen-antibody complexes in oil also differed from the method used here. Comparisons between our results and those of other workers are difficult but, from the observations recorded in the present series of experiments, it is clear that classical delayed skin reactions of the tuberculin type must be characterized by the observation of a pattern of reactivity over a

period of 48-72 hours, rather than by a single observation. A reaction present at 24 hours may be a declining Arthus reaction, a classical delayed reaction or a Jones-Mote reaction and only further observation of the reaction site can assist in their differentiation.

In addition to the possibility of confusing the types of skin reactions illustrated by our experiments, differences may also be present among the strains of animals used. It is well known that variations occur among species in, for example, their reactivity to tuberculin after tuberculous infection or BCG vaccination; and strains of single species may also differ in this respect (Crowle, 1959; Chase, 1959). It may be that the guinea pigs used by different workers differ in their capacity to develop delayed-type hypersensitivity when Mycobacteria are not incorporated in the sensitizing inoculum. There is general agreement that delayed-type hypersensitivity does usually develop when complete adjuvants are used and it is interesting to note that in their more recent work Salvin (1962), Benacerraf and Levine (1962) and Uhr and Pappenheimer (1958) have used complete adjuvants.

Apart from the evidence that in our experiments delayed-type hypersensitivity did not develop in the

guinea pigs unless complete adjuvant was used, our findings are similar in many respects to those of Benacerraf and Gell (1959). Conjugation of proteins with picryl groups appeared to reduce their ability to stimulate the development of Arthus hypersensitivity to the native protein. Animals sensitized with Pic-HSA in complete adjuvant showed almost pure delayed reactions to HSA 7 or 14 days later, when the reactions to the conjugate were mixed. Similarly, in animals sensitized with Pic-OA in adjuvant and skin tested 7 days later, the Arthus component of the reaction to native OA was smaller than that to Pic-OA, but this difference had disappeared in animals tested 14 days after sensitization. Animals sensitized with Pic-HSA in complete adjuvant showed pure Arthus reactivity to Pic-OA, indicating the formation of antibodies without the development of delayed-type hypersensitivity to the hapten. These animals showed strong mixed reactions to Pic-BSA (but no reactions to BSA), a result which suggests that a different specificity was associated with the hapten-serum albumin conjugate. This interpretation is supported by the finding that animals sensitized with Pic-BSA did not show either type of reactivity to BSA despite the occurrence in these animals of strong mixed reactions to the conjugate and of Arthus reactions to Pic-OA.

Conjugation of albumins with picryl groups thus seems to have the following effects on their sensitizing capacity in guinea pigs; (a) Arthus hypersensitivity to the proteins appears later; (b) Arthus hypersensitivity develops to the picryl groups; (c) the complex has a separate specificity not associated with either the picryl group or the protein.

The main effect of complexing the antigen with antibody was to delay the appearance of Arthus hypersensitivity, whether the complex was injected in oil or complete adjuvant. This, too, is in partial agreement with the results of other workers (Uhr, Salvin and Pappenheimer, 1957; Sell and Weigle, 1959).

Further work is necessary to determine the nature of Jones-Mote reactions and their relationship to Arthus and classical delayed reactions. It is possible that Jones-Mote reactions might be due to antibody appearing in the circulation after the intradermal injection of antigen but during the period of observation of the skin test site. If small quantities of antigen persisted at the site of the skin test for 12 to 30 hours, antibody entering the circulation at this time could react with the antigen to give a cutaneous lesion similar to the Arthus

reaction in its brevity though not in its time of onset. In preliminary experiments not described above, this possibility was investigated by titrating the sera of guinea pigs for antibody before sensitization with antigen-antibody complexes, before skin testing (7 days) and after skin testing (10 days). There was, in general, a slight rise in titre during the period of the skin test but in individual animals there was no correlation between this rise and the size of either the Jones-Mote reactions or the Arthus reactions at the second skin test. The results are compatible with the hypothesis above but are also compatible with other interpretations.

Despite the morphological differences between Jones-Mote and classical delayed reactions, the possibility cannot be excluded that the former are, in fact, manifestations of very mild state of delayed-type hypersensitivity. This would imply that they differ in degree rather than in kind from the reactions which we have considered to be those of classical delayed-type hypersensitivity. The question still remains open, whether delayed-type hypersensitivity can be a stage in antibody production in animals sensitized without adjuvants containing Mycobacteria. If this is so, it may be that antibody produced subsequently may suppress the manifestations of a mild degree of delayed-type

hypersensitivity in a manner similar to the enhancement of tumour or skin homografts (Kaliss, 1958; Nelson, 1962), or it may be that the production of "sensitized cells" is completely replaced by the production of serum antibody. Studies in progress on the reactions of cells from hypersensitive guinea pigs to antigens in vitro may help to clarify the issue.

Whether or not the Jones-Mote reactions reflect a state of mild delayed-type hypersensitivity, it is clear from these experiments that the sensitization of guinea pigs with antigen in complete Freund's adjuvant (containing Mycobacteria) regularly induces the capacity to respond to skin tests with reactions which appear to contain both Arthus and delayed components. It is also clear that modification of the antigen may depress its capacity to induce Arthus hypersensitivity.

SUMMARY

Guinea pigs sensitized with ovalbumin
injected subcutaneously or into the footpads or
incomplete adjuvant or intradermally in saline showed
Jones-Matta reactions when skin tested 1 week later.

B. THE CUTANEOUS REACTIVITY OF GUINEA PIGS TO

PURE PROTEIN ANTIGENS

The reactions on a series of animals of similar
intensity in all groups of animals. Mixed Arthus and
delayed-type hypersensitivity resulted from

sensitization with ovalbumin in complete adjuvant.

PAPER II.

THE EFFECTS OF DIFFERENT ROUTES OF INJECTION

OF ANTIGEN AND ADJUVANT

Animals injected subcutaneously. Maximal Arthus and
delayed skin reactions occurred only in animals sensitized
with ovalbumin emulsified in complete adjuvant. (10/10)
Both components of the reactions were diminished in
animals in which the antigen and ovalbumin were
injected separately.

SUMMARY

Guinea pigs sensitized with ovalbumin injected subcutaneously or into the footpads in incomplete adjuvant or intradermally in saline showed Jones-Mote reactions when skin tested 1 week later and Arthus reactions when skin tested 2 weeks later. The reactions on each occasion were of similar intensity in all groups of animals. Mixed Arthus and delayed-type hypersensitivity resulted from sensitization with ovalbumin in complete adjuvant injected either subcutaneously or into the footpads. The delayed reactions were slightly less intense in animals injected subcutaneously. Maximal Arthus and delayed skin reactions occurred only in animals sensitized with ovalbumin emulsified in complete adjuvant. One or both components of the reactions were diminished in animals in which the adjuvant and ovalbumin were injected separately.

INTRODUCTION

The studies described in the preceding paper showed that guinea pigs developed classical delayed-type hypersensitivity to pure proteins only when the sensitizing injection of protein was incorporated in complete adjuvant (containing Mycobacteria). In these experiments, the sensitizing injections were routinely given into the foot pads.

The route of inoculation of antigen in complete adjuvant has been reported not to affect the degree of delayed-type hypersensitivity in guinea pigs (Uhr, Salvin and Pappenheimer, 1957) but to be of some importance in rabbits (Leskowitz and Waksman, 1960). To reinvestigate this question a comparison has been made between the skin reactions of guinea pigs after sensitization with ovalbumin (OA) injected subcutaneously or into the foot pads in incomplete or complete adjuvant, or intradermally without any adjuvant. The effect on cutaneous reactivity has also been examined of injecting the antigen and the adjuvant separately into different sites at the same time or into the same site at different times.

MATERIALS AND METHODS

The animals, the adjuvants and the method of performing and reading skin tests have been described in the preceding papers. The antigen used was hen ovalbumin (OA) (Sigma); for both sensitization and skin testing a dose of 5 micrograms was used. Injections were made into the hind foot pads or subcutaneously into the flank or intradermally into the flank. Skin tests were carried out 7 and/or 14 days after sensitization. When the sensitizing injections had been made into the flank on one side, the opposite side was used as the skin test site.

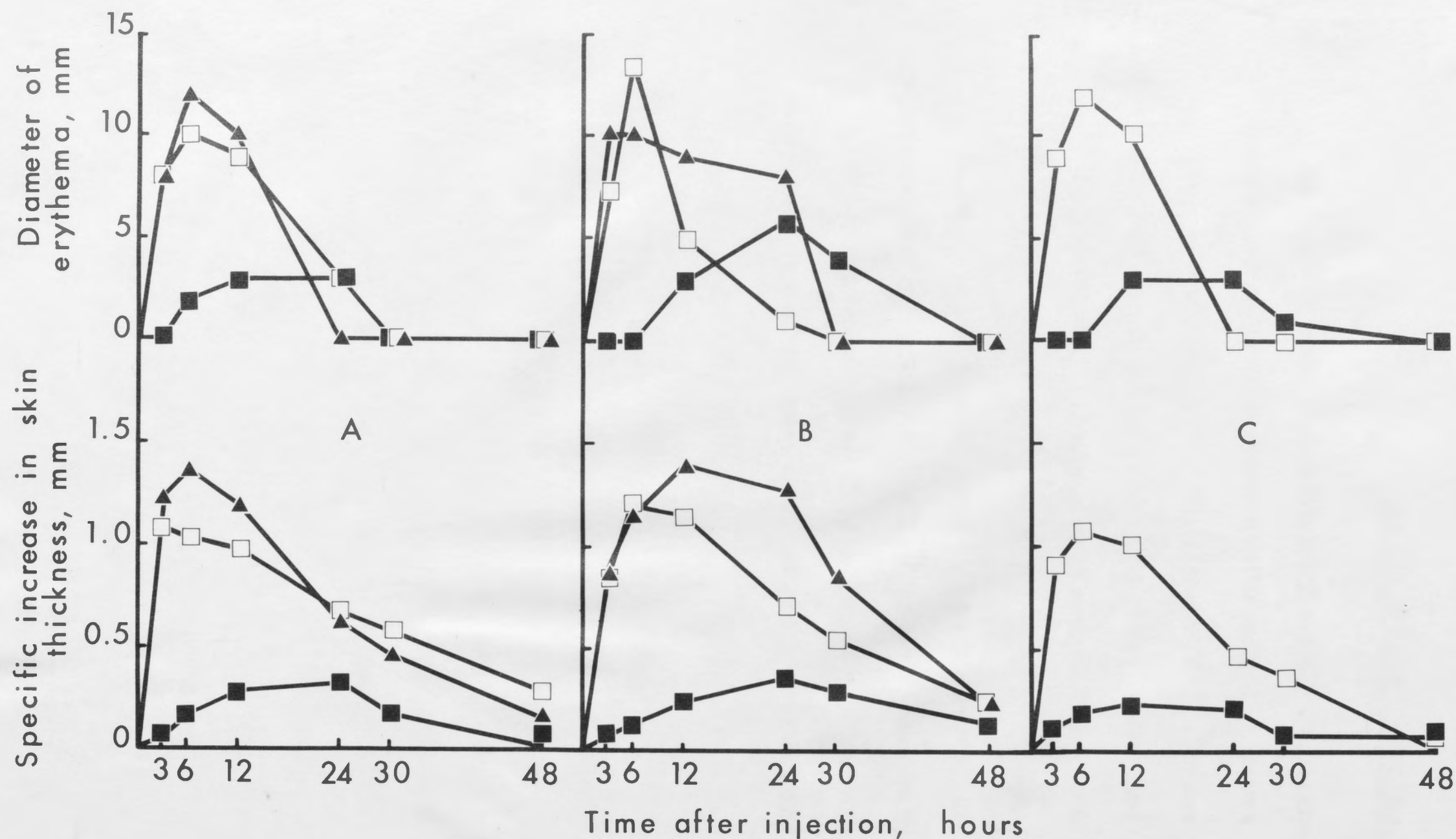


Figure 1. Mean skin reactions to OA in guinea pigs sensitized with OA in oil injected into the footpads (A) or subcutaneously (B); or with OA in saline injected intradermally (C). Reactions to first test 7 days (■—■) or 14 days (▲—▲) after sensitization; and to second test 14 days after sensitization (□—□).

RESULTS

SUBCUTANEOUS, FOOT PAD AND INTRADERMAL INJECTIONS

Guinea pigs were sensitized as follows:

Group A: 5 micrograms OA in oil, injected into the hind foot pads;

Group B: 5 micrograms OA in oil, injected subcutaneously into the flank;

Group C: 5 micrograms OA in saline, injected intradermally into the flank;

Group D: 5 micrograms OA in complete adjuvant, injected into the hind foot pads;

Group E: 5 micrograms OA in complete adjuvant, injected subcutaneously into the flank.

Six or more animals in each group were skin tested 7 and/or 14 days after sensitization. The results of these tests, expressed as mean specific increase in skin thickness and mean diameter of erythema, are shown in Figures 1 and 2.

The animals of Groups A, B, and C tested 7 days after sensitization showed Jones-Mote reactions. When these animals were retested 14 days after sensitization they showed typical Arthus reactions. Animals of Group A tested for the first time 14 days after sensitization also showed

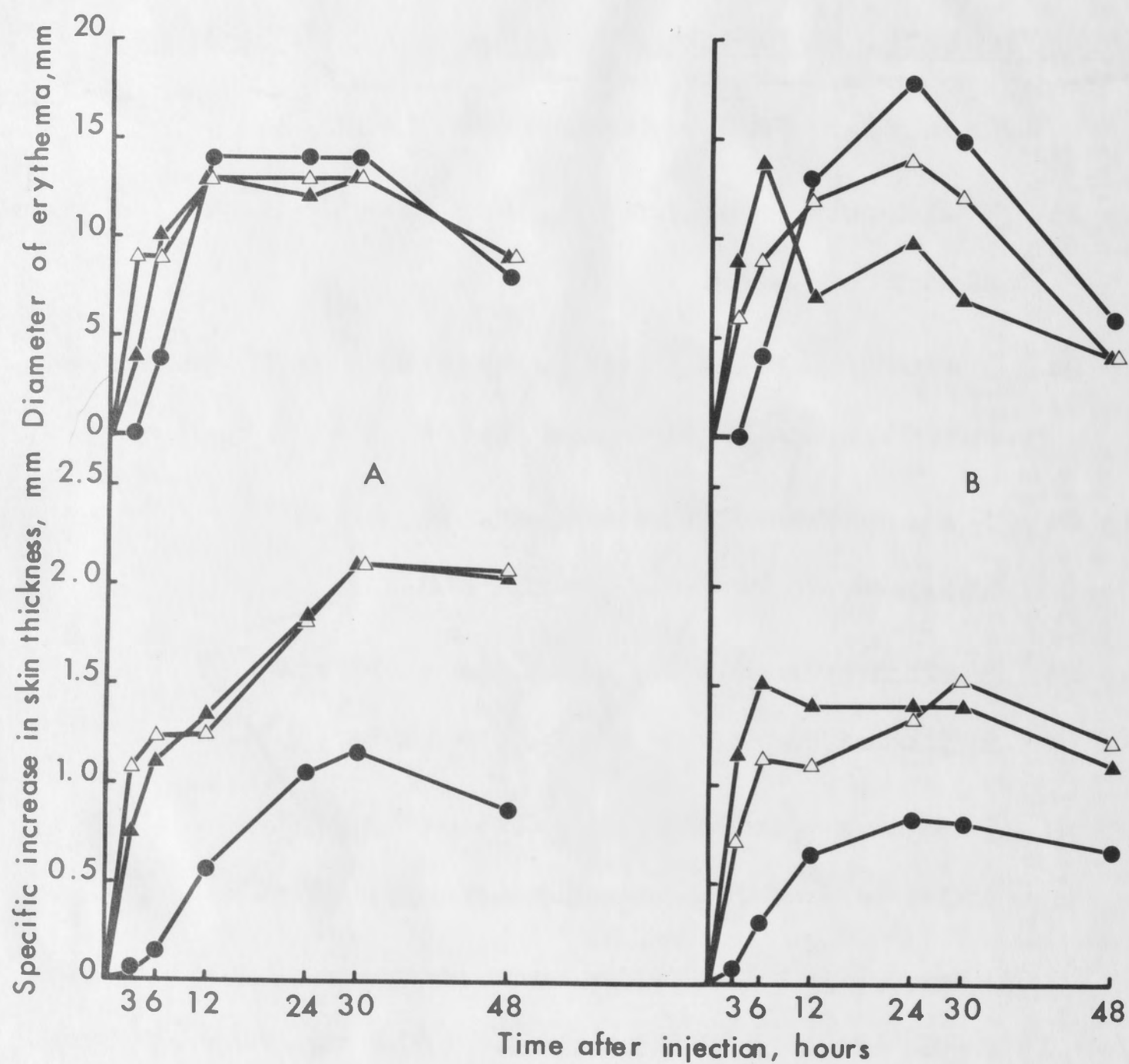


Figure 2. Mean skin reactions to OA in guinea pigs sensitized with OA in complete adjuvant injected into the footpads (A) or subcutaneously (B). Reactions to first test 7 days (●—●) or 14 days (△—△) after sensitization; and to second test 14 days after sensitization (▲—▲).

typical Arthus reactions. In animals of Group B tested for the first time 14 days after sensitization the mean specific increase in skin thickness was maximal at 12 hours and declined only slowly; skin thickening had, however, almost disappeared at 48 hours. These mean results represent a combination of typical Arthus reactions in some animals and Jones-Mote reactions in other animals.

The animals of Groups D and E showed mild classical delayed reactions when tested 7 days after sensitization and mixed Arthus and delayed reactions when tested for the first time or retested 14 days after sensitization. The delayed component of these reactions was slightly less intense in Group D (injected subcutaneously in the flank) than in Group E (injected into the foot pads).

TABLE 1.

Plan for Sensitization of Guinea Pigs by Separate
Injections of Ovalbumin and Complete Adjuvant

Time, days	-7		0		+7	+14
Hind footpad	Left	Right	Left	Right		
Plan A1 (9)	0	0	0.25 ml Ovalbumin	0.25 ml Oval- bumin in adjuvant	Skin test	Skin test
A2 (10)	0	0			0	Skin test
B1 (5)	0	0	0.25 ml Ovalbumin in saline	0.25 ml adjuv- ant	Skin test	Skin test
B2 (5)	0	0			0	Skin test
C1 (6)	0	0.25 ml adjuvant	0	0.25 ml Oval- bumin in saline	Skin test	Skin test
C2 (6)	0		0		0	Skin test
D1 (6)	0	0.25 ml adjuvant	0.25 ml Ovalbumin in adjuvant	0	Skin test	Skin test
D2 (6)	0				0	Skin test

Figures in parenthesis indicate number of animals

Each animal received 5 micrograms ovalbumin

SEPARATE INJECTIONS OF ANTIGEN AND ADJUVANT

A comparison was made of the skin reactions in guinea pigs which had been injected with antigen and complete adjuvant at different sites or at different times. The plan of this experiment is shown in Table 1.

Some of the animals were skin tested at both 7 days and 14 days after sensitization; others were tested only at 14 days. Figure 3 shows the skin reactions (mean specific increase in skin thickness and mean diameter of erythema) in the animals tested for the first time 14 days after sensitization.

When the antigen was injected in saline into one hind foot pad, the adjuvant into the opposite hind foot pad, pure Arthus reactivity resulted. The reactions were similar in intensity to the Arthus component of the mixed reactions in animals sensitized with antigen emulsified with complete adjuvant. The injection of similar doses of antigen into granulomas resulting from injections of adjuvant 7 days previously was followed by the development of less intense Arthus reactivity without any delayed reactivity. When the antigens, emulsified with complete adjuvant, was injected into one hind foot pad 7 days after the injection of adjuvant alone into the opposite hind foot pad, the delayed reactions were as intense as after a single

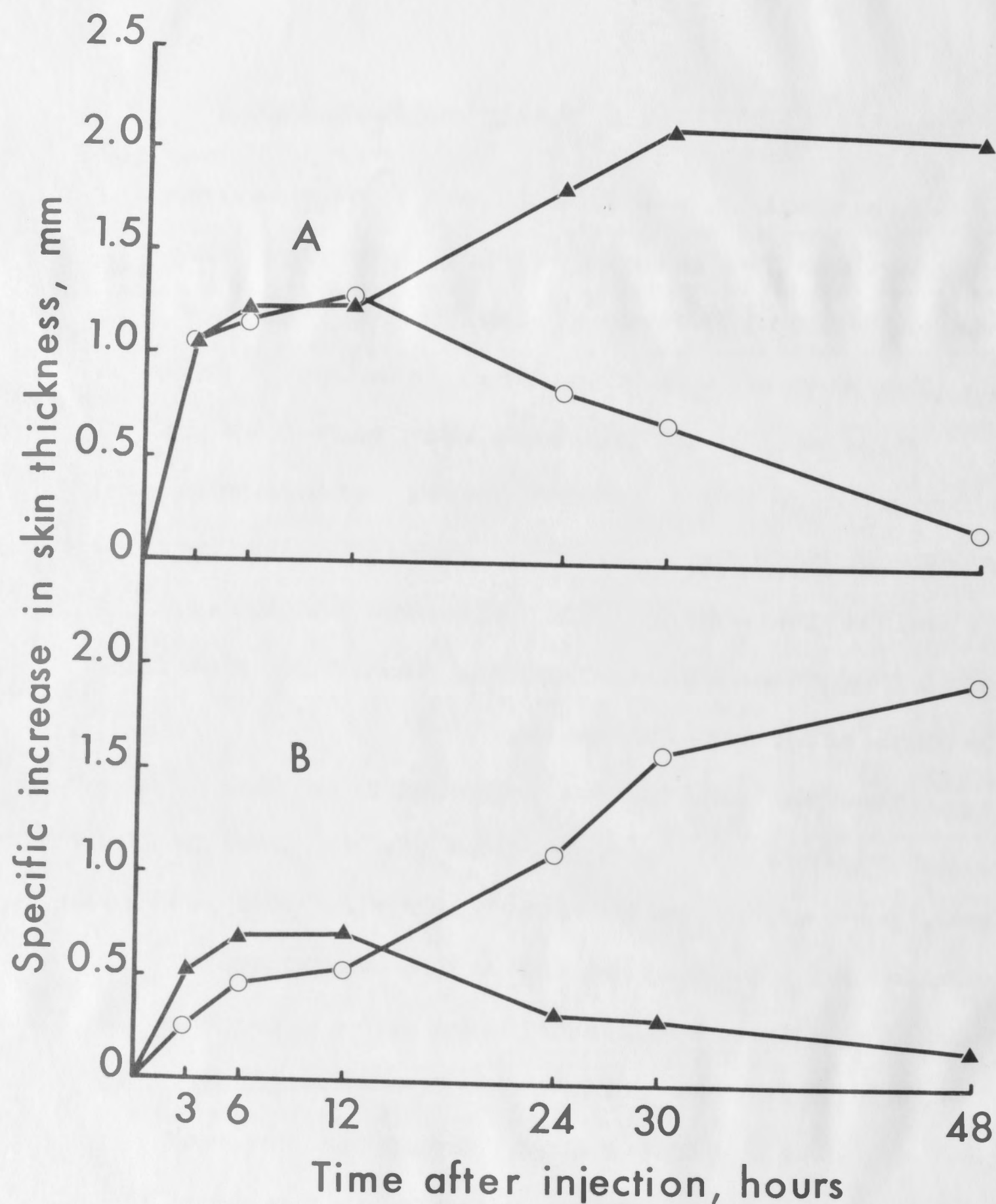


Figure 3. Skin reactions to DA in guinea pigs injected with OA simultaneously with complete adjuvant (A) or 7 days after complete adjuvant (B); OA and adjuvant injected into the same footpads (▲—▲) or opposite footpads (○—○). The animals represented on curve ○—○ of section B received OA in complete adjuvant 7 days after adjuvant alone had been injected into the opposite footpad.

sensitization with antigen emulsified in adjuvant. In this case, however, the Arthus reactions were much less intense.

Similar observations were made on animals tested 7 days after the injection of antigen and retested 7 days later.

TABLE 2.

Summary of Results of Skin Tests after Sensitization
by Different Methods

Method of Sensitization	Skin Reactions					
	7 days			14 days		
	Jones-Mote	Arthus	Delayed	Jones-Mote	Arthus	Delayed
OA in saline, intradermal	+	-	-	-	++	-
OA in oil, subcutaneous	+	-	-	+	++	-
OA in oil, foot pads	+	-	-	-	++	-
OA in adjuvant, subcutaneous	-	-	+	-	++	++
OA in adjuvant, foot pads	-	-	+	-	++	+++
OA in saline, left foot pad; adjuvant, right foot pad, simultaneously	+	-	-	-	++	-
OA in saline, left foot pad; adjuvant, right foot pad, one week earlier	+	-	-	-	+	-
OA in adjuvant, left foot pad; adjuvant, right foot pad, one week earlier	-	-	+	-	+	+++

DISCUSSION

The results of these experiments are summarized in Table 2. Certain clear conclusions can be drawn.

- (1) The reactions to skin tests 7 days after sensitization were the same in the guinea pigs injected with OA in oil into the foot pads or subcutaneously as in those injected with OA in saline intradermally. Since the observations of Jones and Mote (1934) were made on humans sensitized to rabbit serum by intradermal injections, this similarity justifies the use of the eponymous term to describe these transient reactions, whatever the mode of sensitization (cf. Raffel and Newel, 1958).
- (2) The Arthus reactions were of the same intensity whether the animals were sensitized by intradermal injections of OA in saline or subcutaneous or foot pad injections of OA in oil (in which cases the Arthus reactions occurred alone), or by subcutaneous or foot pad injections of OA in complete adjuvant (in which cases they occurred with delayed reactions). Thus neither complete nor incomplete adjuvant enhanced Arthus hypersensitivity.

- (3) Whether OA in complete adjuvant was injected into the foot pads or subcutaneously, delayed-type hypersensitivity was apparent 7 days after sensitization and mixed delayed-type and Arthus hypersensitivity 14 days after sensitization. The delayed reactions were slightly less intense in the animals injected subcutaneously than in those injected in the foot pads. This is similar to the finding of Leskowitz and Waksman (1960) who used rabbits sensitized with bovine serum albumin in complete adjuvant. There is a slight discrepancy between this observation and the report of Uhr et al. (1957) that subcutaneous, intramuscular and intraperitoneal injections of antigen-antibody complexes in complete adjuvant were as effective as injections into the digits of guinea pigs. This apparent discrepancy is probably accounted for by differences in the technique of measuring the intensity of the delayed reactions.
- (4) When OA was injected at the same time as adjuvant but separately into a different foot pad, only Arthus hypersensitivity developed. No delayed-type hypersensitivity developed after the injection of OA

in saline into granulomata resulting from the injection of adjuvant one week earlier. This latter finding was rather unexpected in view of the work of Dienes (1929), who succeeded in inducing delayed-type hypersensitivity to egg albumin in guinea pigs by injecting the protein into large inguinal granulomata resulting from two injections of live or killed tubercle bacilli. It is possible that both the size and cellular composition of the granuloma may affect the result of such injections.

- (5) Injection of complete adjuvant alone into one foot pad one week before the injection of OA in complete adjuvant into the opposite foot pad did not affect the delayed component of the mixed reactions to subsequent skin tests. The Arthus component was, however, smaller than in animals injected with OA in adjuvant without a prior injection of adjuvant alone. This is contrary to the findings of other workers. Janković (1962) reported that the injection of complete adjuvant 10 days before sensitizing injections of protein antigens in complete adjuvant grossly reduced the delayed skin reactions of guinea pigs tested 7 or 17 days later. Kies and Alvord (1958)

reported that the injection of complete adjuvant 30 days before sensitizing injections of spinal cord emulsions in complete adjuvants likewise decreased the incidence and severity of "allergic" encephalomyelitis in guinea pigs. This experimental disease is considered to have a basis in delayed-type hypersensitivity to constituents of nervous tissue (Waksman and Adams, 1962). In these investigations, however, the adjuvant contained much larger quantities of Mycobacteria than did the adjuvant we have used, and the bacteria were killed M. tuberculosis, not M. butyricum. Furthermore, the intervals between the injection of adjuvant and the sensitizing injections were longer. These differences may explain the discrepancy.

The results of the experiments described in the preceding paper showed that classical delayed-type hypersensitivity to pure protein antigens, often accompanied by Arthus hypersensitivity, developed in guinea pigs only when they were sensitized with antigens incorporated in adjuvant containing Mycobacteria. The experiments described in this paper indicate that, in order for delayed-type hypersensitivity to develop, the association between antigen and adjuvant must be an intimate one. Raffel, Arnaud,

Dukes and Huang (1949) found that if antigen was injected into the same site as the adjuvant after an interval of 2 hours, delayed-type hypersensitivity was induced, but if the interval was 24 hours delayed-type hypersensitivity was not induced. Stone and Freund (1959) found that injections of adjuvant at sites close to those of antigen injections made at the same time were effective, but that injections at distant sites were ineffective. Neither these experiments nor the experiments reported here throw much light on the mode of action of adjuvants in promoting the development of delayed-type hypersensitivity. It seems likely that this depends somehow on the way in which antigen is "processed" by cells before it reaches immunologically competent cells (White, Coons and Connolly, 1955).

(Immunology, 6, 264-273, 1963).

SUMMARY

Exudates were induced in the peritoneal cavity of guinea pigs by the injection of glycogen. The content of these exudates was examined after 4 days in normal and hypersensitive animals. In animals sensitized with antigens, the exudates contained a high proportion

C. REACTIONS TO ANTIGEN OF PERITONEAL CELLS OF GUINEA PIGS WITH DELAYED-TYPE HYPERSENSITIVITY

In BCG- to tuberculin, subcutaneous, intravenous or intraperitoneal injection of tuberculin resulted in a profound fall in the macrophage content of the exudate. This effect was

PAPER III.

THE LOSS OF MACROPHAGES FROM PERITONEAL EXUDATES FOLLOWING THE INJECTION OF ANTIGENS INTO GUINEA PIGS WITH DELAYED-TYPE HYPERSENSITIVITY

tuberculin into guinea pigs with Arthus hypersensitivity to tuberculin. Ovalbumin injected intraperitoneally into guinea pigs with Arthus hypersensitivity to ovalbumin also resulted in a marked fall

in the macrophage content of peritoneal exudates, but had no effect on the peritoneal macrophages of animals with pure Arthus hypersensitivity. Bacterial endotoxin injected intraperitoneally caused a similar fall in the macrophage contents of exudates of both normal and BCG-sensitized animals. It is concluded that this fall in macrophage

SUMMARY

Exudates were induced in the peritoneal cavities of guinea pigs by the injection of glycogen. The cell content of these exudates was examined after 4 days in normal and hypersensitive animals. In animals uninjected with antigens, the exudates contained a high proportion of macrophages, together with lymphocytes and polymorphs. In BCG vaccinated animals, with delayed-type hypersensitivity to tuberculin, subcutaneous, intravenous or intraperitoneal injection of tuberculin resulted in a profound fall in the macrophage content of the exudates. This effect was apparent within an hour of intraperitoneal injection and occurred with very small doses of tuberculin. No such effect occurred after the intraperitoneal injection of tuberculin into guinea pigs with Arthus hypersensitivity to tuberculin. Ovalbumin injected intraperitoneally into guinea pigs with mixed delayed-type and Arthus hypersensitivity to ovalbumin also resulted in a marked fall in the macrophage content of peritoneal exudates, but had no effect on the peritoneal macrophages of animals with pure Arthus hypersensitivity. Bacterial endotoxin injected intraperitoneally caused a similar fall in the macrophage contents of exudates of both normal and BCG vaccinated animals. It is concluded that this loss of macrophages

from peritoneal exudates following the injection of antigen is the consequence of an immunological reaction which is a manifestation of a state of delayed-type hypersensitivity.

Since no convincing evidence has been produced for the involvement of specific serum factors in the reaction, it is generally assumed that the specific reactivity is strictly cell-associated. However, "cell-fixed" or "sessile" antibodies have not been demonstrated in tuberculin type hypersensitivity. The fact that hypersensitivity can be transferred to normal animals by lymphoid cells from hypersensitive animals (Chase, 1945) indicates that the specific change responsible for the hypersensitive state probably arises in lymphoid tissue. But passive transfer experiments have not shown definitely whether the transferred cells are themselves specifically "sensitive" in any way to tuberculin, or whether they carry or synthesize some specific antibody-like substance which reacts with the antigen.

Ignorance of the mechanism of tuberculin-type reactions persists in spite of a great deal of experimental work on the effects of antigens on cells from normal and hypersensitive animals in vitro. Rich and Lewis (1937) reported that tuberculin was specifically toxic for the

INTRODUCTION

The mechanism of the tuberculin reaction and of other delayed allergic reactions remains a mystery. Since no convincing evidence has been produced for the involvement of specific serum factors in the reaction, it is generally assumed that the specific reactivity is strictly cell-associated. However, "cell-fixed" or "sessile" antibodies have not been demonstrated in tuberculin type hypersensitivity. The fact that hypersensitivity can be transferred to normal animals by lymphoid cells from hypersensitive animals (Chase, 1945) indicates that the specific change responsible for the hypersensitive state probably arises in lymphoid tissue. But passive transfer experiments have not shown definitely whether the transferred cells are themselves specifically "sensitive" in any way to tuberculin, or whether they carry or synthesise some specific antibody-like substance which reacts with the antigen.

Ignorance of the mechanism of tuberculin-type reactions persists in spite of a great deal of experimental work on the effects of antigens on cells from normal and hypersensitive animals in vitro. Rich and Lewis (1932) reported that tuberculin was specifically toxic for the

cells of spleen explants from tuberculin-sensitive guinea pigs, inhibiting the migration of the cells from the tissue fragments. Other workers have reported similar findings (see reviews by Boyden, 1958; Raffel, 1954; Waksman, 1958). However, the technique has not given clear-cut results in the hands of all investigators and there is some question whether the effect described by Rich and Lewis has any direct connection with the tuberculin reaction as seen in the skin test. The concentrations of antigen required to inhibit the migration of cells from hypersensitive animals are very high compared with the concentrations which will give a positive skin test in a hypersensitive animal. Moreover, the dose of antigen required to inhibit the migration of "sensitive" cells is usually only about ten times less than the amount which will produce a similar effect on cells from normal animals. Indeed, it has been suggested that this in vitro reaction may be quite non-specific, due perhaps to a greater susceptibility of the cells from the hypersensitive animal to toxic agents in general (Lasfargues, Boquet and Delaunay, 1947). This idea receives some support from the work of Tunçman and Packalén (1959), who report that cell migration from spleen explants taken from tuberculin sensitive guinea pigs is inhibited by culture filtrates of E. coli at concentrations which do not affect the migration of

normal cells.

Analogous studies in vivo have also failed to throw much light on the mechanism of the tuberculin reaction. Holst (1921) injected tuberculin intraperitoneally into normal and hypersensitive guinea pigs. The exudate cells harvested later from the tuberculin sensitive animals were mostly dead, while those from normal animals were living. Stewart, Long and Bradley (1926) injected tuberculin into pleural exudates which had been induced by a previous injection of broth; at intervals afterwards they withdrew samples and stained them supravitaly. Twenty four hours after the tuberculin had been injected, nearly all the cells from tuberculous guinea pigs were dead, while the majority of the cells from normal animals were living. The amounts of tuberculin used to produce these effects were again very high compared with the amounts necessary to give a positive skin test; information was not provided on the difference between the effective dose of tuberculin in hypersensitive guinea pigs and that which would produce a similar cytotoxic effect in normal animals. These experiments do not show whether the leucocytes are directly damaged by tuberculin or whether they are damaged by some product of a reaction between tuberculin and components of body fluids or other cell types (e.g., serosal cells).

The observations reported below arose from the chance observation that the subcutaneous injection of tuberculin caused the almost complete disappearance, within a few hours, of macrophages from glycogen-induced peritoneal exudates in hypersensitive guinea pigs (Nelson and Boyden, 1961). It was found that direct intraperitoneal injection of tuberculin produced the same effect: in this case the reaction could be observed with as little as 0.1 microgram of tuberculin. One thousand times this quantity failed to produce a similar response in normal guinea pigs. The reaction appears to differ in several respects from the other effects of tuberculin mentioned above: we have considered it worthy of some detailed investigation as another possible clue to the mechanism of tuberculin hypersensitivity. The present paper describes some basic characteristics of this effect of tuberculin on exudate cells of tuberculin sensitive guinea pigs.

MATERIALS AND METHODS

Animals. Albino guinea pigs of both sexes were used. They were supplied by the Animal Breeding Establishment, Australian National University and weighed 400 to 700 gm at the time of use.

Exudates. Oyster glycogen (British Drug Houses) was dissolved in normal saline to a concentration of 0.02 mg per ml and sterilized by autoclaving. Four days before each experiment guinea pigs were injected intraperitoneally with 10 ml of this solution.

Antigens. BCG was obtained from the Commonwealth Serum Laboratories, Melbourne.

Purified Protein Derivative (PPD) of tuberculin was supplied by the State Serum Institute, Copenhagen, Denmark. Batch RT 23 was used in these experiments.

Tuberculin (unheated) was prepared from filtrates of cultures of human virulent tubercle bacilli. The cultures were Seitz filtered twice, concentrated by ultrafiltration and then lyophilized.

Ovalbumin, twice crystallized, was obtained from Nutritional Biochemicals Corporation, Cleveland, O.

Endotoxin, derived from E. coli, was obtained from Difco Laboratories, Detroit, Mich.

For skin testing, the antigens were dissolved in saline and injected in 0.1 ml quantities. For intraperitoneal, intravenous and subcutaneous injection, they were

dissolved in Hanks' balanced salt solution and injected in a volume of 1 ml.

Immunization. The guinea pigs were divided into four groups. They were injected as follows:

Group 1: No injection (controls).

Group 2: An intradermal injection of 0.1 mg BCG 4 to 6 weeks before use.

Group 3: A single subcutaneous injection of 0.1 mg tuberculin (unheated) in Freund's incomplete adjuvant (Difco) 4 to 5 weeks before use; or subcutaneous injections of 1 mg tuberculin (unheated) in saline at weekly intervals.

Group 4: 5 micrograms of ovalbumin in Freund's incomplete adjuvant, injected into the digits of the hind feet, followed by 1 or 2 injections of 5 micrograms intradermally 1 or 2 weeks later. These latter injections were made for the purpose of skin testing, but served also as secondary stimuli to antibody production. The animals were used 4 weeks after the first injection.

Group 5: 5 micrograms of ovalbumin in Freund's complete adjuvant containing

Mycobacteria (Difco), injected into the digits of the hind feet or into the hind foot pads. About half these animals also received 1 or 2 injections of 5 micrograms intradermally 1 or 2 weeks later, for the purpose of skin testing. The animals were used 4 weeks after the first injection.

Skin testing. A saline solution of the antigen (5 or 10 micrograms in 0.1 ml) was injected intradermally into the previously shaved skin of the flank. The reactions were observed 3, 6, 12, 24, 30, 48 and sometimes 72 hours later. Before injection and at each reading afterwards the skin thickness at the injection site was measured by means of skin calipers (Schnelltaster, System Kroplin, Type A.02 T, H.C. Kroplin, G.m.b.H., Schlüchtern, Hessen, Germany). The results are expressed in the Figure below as "specific increase in skin thickness". This represents the average increase, in mm, in skin thickness in sensitive animals, less the average increase in skin thickness in normal animals tested at the same time. In addition, the diameter of the area of erythema at the injection site was measured and the degree of erythema arbitrarily graded.

Harvest of Exudates. The fluid used to wash out the peritoneal cavities consisted of 3% normal guinea pig serum in Hanks' balanced salt solution containing 5 units

of heparin per ml. Animals were killed by exsanguination under ether anaesthesia and stretched supine on a board. The abdominal skin was reflected and 20 ml of fluid introduced into the peritoneal cavity. After thorough mixing, the board was tilted and the fluid was drained off through a pubic incision into a large test tube.

Estimation of cell content of exudates. Total cell counts were made on the undiluted washed out fluid in a haemocytometer. For differential cell counts, 2 to 3 ml of fluid was centrifuged lightly, the supernatant removed and the cells resuspended in a few drops of 50% guinea pig serum. A drop of this suspension was smeared between coverslips, dried in air and stained with Wright's stain. The distribution of cell types in such smears was even. Under oil immersion, 200 or 300 cells were counted and classified as follows:

Macrophages: Large cells with abundant cytoplasm and with a single (or rarely a double) nucleus which contains pale diffuse chromatin and which is frequently indented;

Lymphocytes: Small cells with very little cytoplasm and with a densely staining nucleus containing smaller and denser concentrations of chromatin;

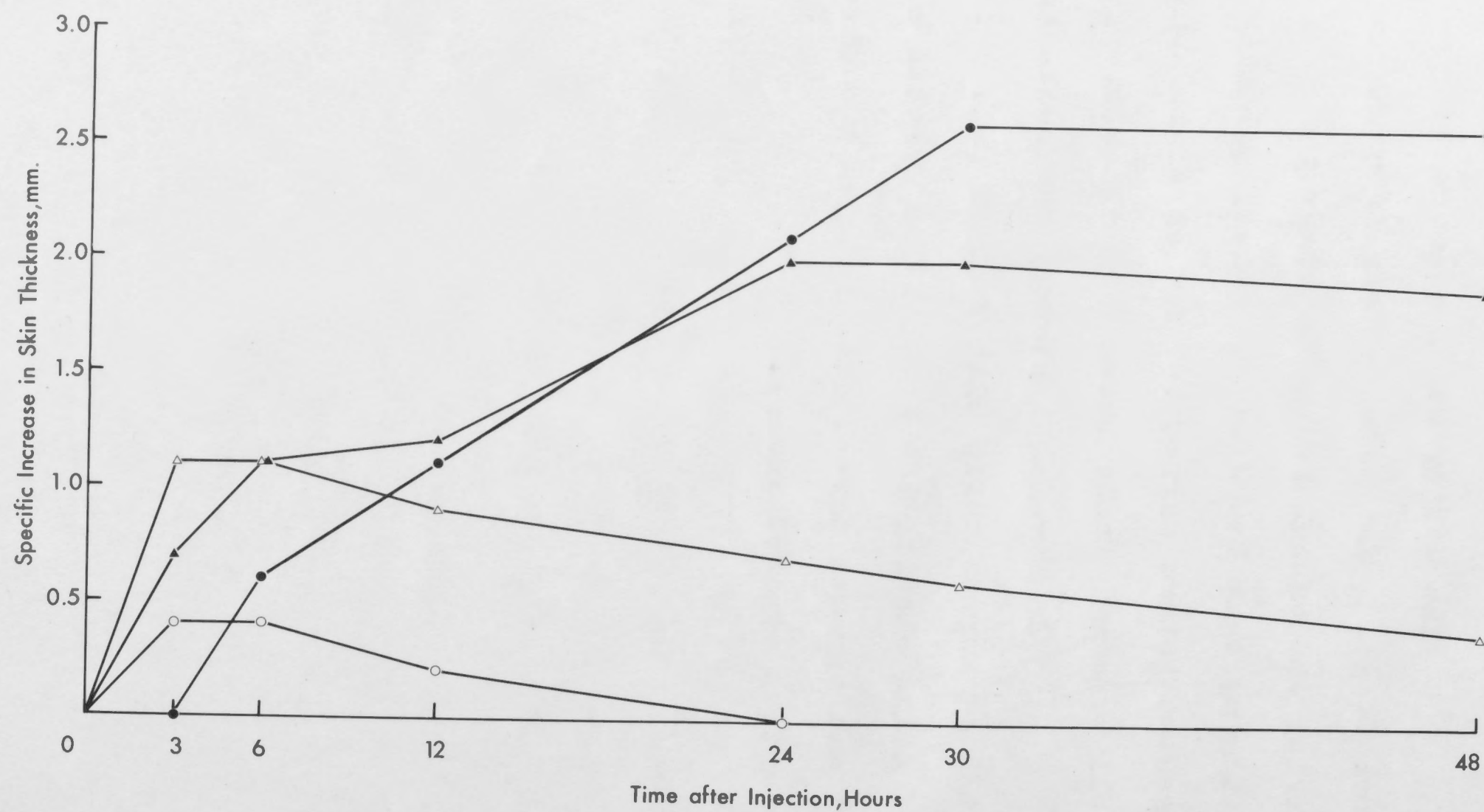
Polymorphs: Polymorphonuclear cells with granular cytoplasm; differentiation between eosinophils, neutrophils

and basophils was not made in counting.

The differential count was expressed as a percentage. The total number of cells in the peritoneal cavity was calculated as 20 times the cell count per ml, assuming that there was a minimal quantity of fluid present before harvest. The absolute numbers of each cell type were calculated from these two figures.

In several instances the cells of exudates were stained supravitaly with Neutral Red. The percentage of cells showing the Neutral Red rosette characteristic of macrophages agreed closely with the differential count on stained smears.

Figure 1. Results of skin tests (mean specific increases in skin thickness). Group 2 (BCG-vaccinated) (●—●) and Group 3 (immunized with unheated tuberculin (○—○)), tested with 10 micrograms PPD. Group 4 (immunized with OA in oil) (△—△) and Group 5 (immunized with OA in complete adjuvant) (▲—▲) tested with 5 micrograms OA.



RESULTSSkin reactions.

The characteristics of the skin reactions of the four groups of guinea pigs used in the experiments described below are shown in Figure 1. The antigens used in the skin tests were as follows:

for Group 2 (BCG-vaccinated) - PPD, 10 micrograms

" " 3 (immunized with tuberculin (unheated)) -
PPD, 10 micrograms

" " 4 (immunized with ovalbumin in incomplete
adjuvant) - ovalbumin, 5 micrograms

" " 5 (immunized with ovalbumin in complete
adjuvant) - ovalbumin, 5 micrograms

The results of the skin tests indicate that the BCG-vaccinated guinea pigs (Group 2) gave typical delayed reactions, showing very little thickening at 3-6 hours and maximum thickening at 24-48 hours.

The animals injected with tuberculin (unheated) (Group 3) or with ovalbumin in incomplete adjuvant (Group 4) had typical Arthus reactions, with maximum thickening at 3-6 hours; by 24 hours the skin thickness was almost normal. There was no difference in the skin reactions between those animals of Group 3 immunized by multiple

TABLE 1.

Cell Contents of Peritoneal Exudates of Normal and BCG-Vaccinated
Guinea Pigs after Subcutaneous Injections of PPD

Treatment of Animals, Time between Injection and Harvest and Number of Animals	Absolute Cell Count \pm Standard Error $\times 10^{-4}$		
	Macrophages	Lymphocytes	Polymorphs
Group 1 - Normal			
Uninjected 38	1514 \pm 110	900 \pm 128	198 \pm 34
PPD 400 micrograms, 2 hrs. 5	2419 \pm 586**	2276 \pm 571	162 \pm 119
PPD 400 micrograms, 6 hrs. 5	2434 \pm 793**	1423 \pm 536	367 \pm 208
Group 2 - BCG-Vaccinated			
Uninjected 26	1782 \pm 131	1370 \pm 172	152 \pm 45
PPD 400 micrograms, 2 hrs. 7	1489 \pm 345	1182 \pm 232	754 \pm 300**
PPD 400 micrograms, 6 hrs. 7	177 \pm 44**	481 \pm 121	504 \pm 117**

Statistical significance of differences between cell contents of exudates of PPD-injected and uninjected animals

** $P < 0.01$

* $P = 0.01-0.05$

Others are not significant ($P > 0.05$)

injections of tuberculin (unheated) in saline and those immunized by a single injection of the antigen in incomplete adjuvant. The pooled results are shown in the graph.

All the animals injected with ovalbumin in complete adjuvant (Group 5) gave mixed Arthus and delayed reactions to ovalbumin. Although the graph shows average results, the skin thickening in individual animals followed this course in time.

In Groups 3, 4 and 5, the skin tested animals were used in the experiments on peritoneal exudates, along with similarly immunized animals which had not been skin tested. In the case of Group 2 only animals which had not been skin tested were used in these experiments.

The effect of subcutaneous and intravenous injections of tuberculin (PPD) on the cell content of peritoneal exudates.

Normal (Group 1) and BCG-vaccinated (Group 2) guinea pigs were injected intraperitoneally with glycogen. Four days later 400 micrograms of PPD was injected subcutaneously into the right inguinal region. The exudates were harvested and counted 2 or 6 hours later. Table 1 shows the cell contents of the exudates compared with those of control animals not injected with tuberculin.

TABLE 2.

Macrophage Contents of Peritoneal Exudates of Guinea Pigs 4 Hours after the
Intravenous Injection of 400 micrograms of PPD

Group of Animals and Number in Groups	Total Macrophage Count \pm Standard Error $\times 10^{-4}$
Group 1 - Normal 7	1719 \pm 232
Group 2 - BCG-Vaccinated 6	178 \pm 76

The most striking feature of these results is the fact that the average macrophage count in the sensitive animals 6 hours after the injection of tuberculin is lower by 90% than the average count in the controls. The macrophage counts of exudates of sensitive animals only 2 hours after the injection of tuberculin were not significantly different from the controls.

In contrast, the average macrophage counts in the normal animals 2 and 6 hours after the injection of tuberculin were somewhat higher than the average counts in the non-injected animals. The significance of these higher counts (and of the high lymphocyte counts in normal animals 2 hours after the injection of tuberculin) is not clear, especially since no similar tendency was seen in other experiments in which tuberculin was injected intraperitoneally or intravenously (see below).

To exclude the possibility that the low macrophage counts might be due to the accidental injection of tuberculin into the peritoneal cavity, some guinea pigs were injected with PPD intravenously. The results, which are shown in Table 2, were similar to those described above, the average macrophage count of the hypersensitive animals being only 10% of that of the normal animals injected with PPD. Similar results were obtained when PPD was injected subcutaneously into the axillary region.

TABLE 3.

Cell Contents of Peritoneal Exudates of Normal, BCG-Vaccinated and Tuberculin-Immunized Guinea Pigs 4 hours after Intraperitoneal Injection of 10 micrograms PPD or 1 ml. Hanks' Solution

Group of Animals, Material Injected and Number in Group		Absolute Cell Counts \pm Standard Error x 10 ⁻⁴		
		Macrophages	Lymphocytes	Polymorphs
Group 1 - Normal				
Uninjected	38	1514 \pm 110	900 \pm 128	198 \pm 34
Hanks' Solution, 1 ml	8	1133 \pm 191	819 \pm 126	2257 \pm 692
PPD, 10 micrograms	20	1094 \pm 95	858 \pm 129	2043 \pm 202
Group 2 - BCG-Vaccinated				
Uninjected	26	1782 \pm 131	1370 \pm 172	152 \pm 45
Hanks' Solution, 1 ml	6	1471 \pm 231	1510 \pm 306	1909 \pm 453
PPD, 10 micrograms	28	117 \pm 22**	848 \pm 153	1800 \pm 175
Group 3 - Immunized with Tuberculin (unheated)				
Uninjected	4	2112 \pm 420	553 \pm 63	305 \pm 145
Hanks' Solution, 1 ml	8	1383 \pm 235	1042 \pm 250	1045 \pm 224
PPD, 10 micrograms	8	1703 \pm 303	585 \pm 88	3025 \pm 462**

Statistical significance of difference between cell contents of exudates of animals injected with Hanks' Solution and those injected with PPD:-

** $P \leq 0.01$

* $P = 0.01-0.05$

Others are not significant ($P > 0.05$)

The effect of intraperitoneal injections of tuberculin and of Hanks' solution on the cell content of peritoneal exudates.

Guinea pigs of Group 1 (normal), Group 2 (BCG-vaccinated) and Group 3 (immunized with unheated tuberculin) were injected intraperitoneally with glycogen 4 days before use. Some received no further injection, while others received either 1 ml of Hanks' solution or 10 micrograms of PPD in 1 ml of Hanks' solution intraperitoneally 4 hours before harvest. Table 3 shows the cell content of the peritoneal exudates of these animals.

Again, the striking feature of these results is the very low content of macrophages in the exudates of animals with delayed-type hypersensitivity to tuberculin (Group 2), after the injection of tuberculin. No such effect was observed in either the normal animals (Group 1) or those with Arthus sensitivity to tuberculin (Group 3).

Hanks' solution alone caused a significant rise in the polymorph content of exudates. The injection of tuberculin resulted in similarly increased polymorph counts in the BCG-vaccinated animals (Group 2) and even higher counts in the animals with Arthus sensitivity to tuberculin (Group 3).

The effect of intraperitoneal injections of ovalbumin on the cell content of peritoneal exudates.

The following experiment was designed to determine the effect of intraperitoneal injections of ovalbumin on the cell counts of exudates in normal guinea pigs and in guinea pigs with delayed-type hypersensitivity to tuberculin (Group 2), with Arthus hypersensitivity to ovalbumin (Group 4) or with mixed Arthus and delayed-type hypersensitivity to ovalbumin (Group 5).

As before, the animals were injected intraperitoneally with glycogen 4 days before use.. On the day of the experiment some animals from each group were injected intraperitoneally with 10 micrograms of ovalbumin in Hanks' solution. Control animals received 1 ml of Hanks' solution. Four hours later the exudates were harvested and the cells were counted. The results are presented in Table 4.

It may be noted that the average macrophage count for control animals in Group 5 is about twice the average count for the controls in either of the other groups. This difference is statistically significant ($p = 0.01-0.05$). It seems probable that the higher counts in Group 5 are the consequence of the non-specific stimulating effect of complete Freund's adjuvant on the reticulo-endothelial

TABLE 4.

Cell Contents of Peritoneal Exudates of Normal, BCG-Vaccinated and Ovalbumin-Immunized Guinea Pigs 4 hours after Intraperitoneal Injection of 10 micrograms Ovalbumin or 1 ml Hanks' Solution

Group of Animals, Material Injected and Number in Group	Absolute Cell Counts \pm Standard Error $\times 10^{-4}$		
	Macrophages	Lymphocytes	Polymorphs
Group 1 - Normal			
Hanks' Solution, 1 ml	8	819 \pm 126	2257 \pm 692
Ovalbumin, 10 micrograms	10	1124 \pm 222	1118 \pm 192
Group 2 - BCG-Vaccinated			
Hanks' Solution, 1 ml	6	1510 \pm 306	1909 \pm 453
Ovalbumin, 10 micrograms	9	1178 \pm 245	1477 \pm 315
Group 4 - with Arthus-type hypersensitivity to Ovalbumin			
Hanks' Solution, 1 ml	12	760 \pm 139	1470 \pm 278
Ovalbumin, 10 micrograms	13	873 \pm 139	1226 \pm 380
Group 5 - with Mixed Delayed and Arthus-type hypersensitivity to Ovalbumin			
Hanks' Solution, 1 ml	6	2871 \pm 1250	2519 \pm 514
Ovalbumin, 10 micrograms	13	992 \pm 256	1682 \pm 222

Statistical significance of difference between cell contents of exudates of animals injected with Hanks' Solution and those injected with ovalbumin

** $P \leq 0.01$

* $P = 0.01-0.05$

Others are not significant ($P > 0.05$)

system.

The intraperitoneal injection of ovalbumin into the guinea pigs with some delayed-type hypersensitivity to this antigen (Group 5) has apparently resulted in a marked drop in the macrophage content of the exudates: the average macrophage count in these animals is 85% lower than that in animals of the same group injected with Hanks' solution only.

After the intraperitoneal injection of ovalbumin into animals of Group 4, which had typical Arthus hypersensitivity but no delayed reactivity to the antigen, the average macrophage count was slightly lower (by 27%) than the count in control animals injected with Hanks' solution. This difference is not statistically significant and in any case is much smaller than the difference between the two counts in Group 5 animals.

The results of this experiment are therefore consistent with those of the last and are compatible with the interpretation that the disappearance of macrophages from the exudates following intraperitoneal injection of antigen is associated with the state of delayed-type hypersensitivity.

The effect of different doses of tuberculin on the cell content of peritoneal exudates of guinea pigs with delayed-type hypersensitivity to tuberculin.

Normal (Group 1) and BCG-vaccinated (Group 2)

TABLE 5.

Cell Contents of Peritoneal Exudates of Normal and BCG-Vaccinated Guinea Pigs 4 hours after Intraperitoneal Injection of Different Doses of PPD

Group of Animals, Material Injected, Number of Animals in Group		Absolute Cell Count \pm Standard Error $\times 10^{-4}$		
		Macrophages	Lymphocytes	Polymorphs
Group 1 - Normal				
Hanks' Solution, 1 ml	8	1133 \pm 191	819 \pm 126	2257 \pm 692
PPD 1 microgram	6	1172 \pm 182	1120 \pm 386	1981 \pm 523
PPD 10 micrograms	20	1094 \pm 95	858 \pm 129	2043 \pm 202
PPD 100 micrograms	9	1030 \pm 182	755 \pm 195	3788 \pm 265*
Group 2 - BCG-Vaccinated				
Hanks' Solution, 1 ml	6	1471 \pm 231	1510 \pm 306	1909 \pm 453
PPD 0.1 micrograms	6	277 \pm 22**	549 \pm 89*	1091 \pm 176
PPD 1 microgram	6	166 \pm 58**	956 \pm 235	2072 \pm 516
PPD 10 micrograms	28	117 \pm 22**	848 \pm 153	1800 \pm 175
PPD 100 micrograms	9	90 \pm 22**	388 \pm 84**	3319 \pm 513*

Statistical significance of differences between cell counts of exudates of animals injected with Hanks' Solution and those injected with PPD;

** $P \leq 0.01$

* $P = 0.01-0.05$

Others are not significant ($P > 0.05$)

guinea pigs with glycogen induced exudates were injected intraperitoneally with 0.1, 1, 10 or 100 micrograms of PPD 4 hours before the harvest of exudates. The cellular composition of these exudates is compared in Table 5 with the composition of exudates of animals injected with Hanks' solution alone.

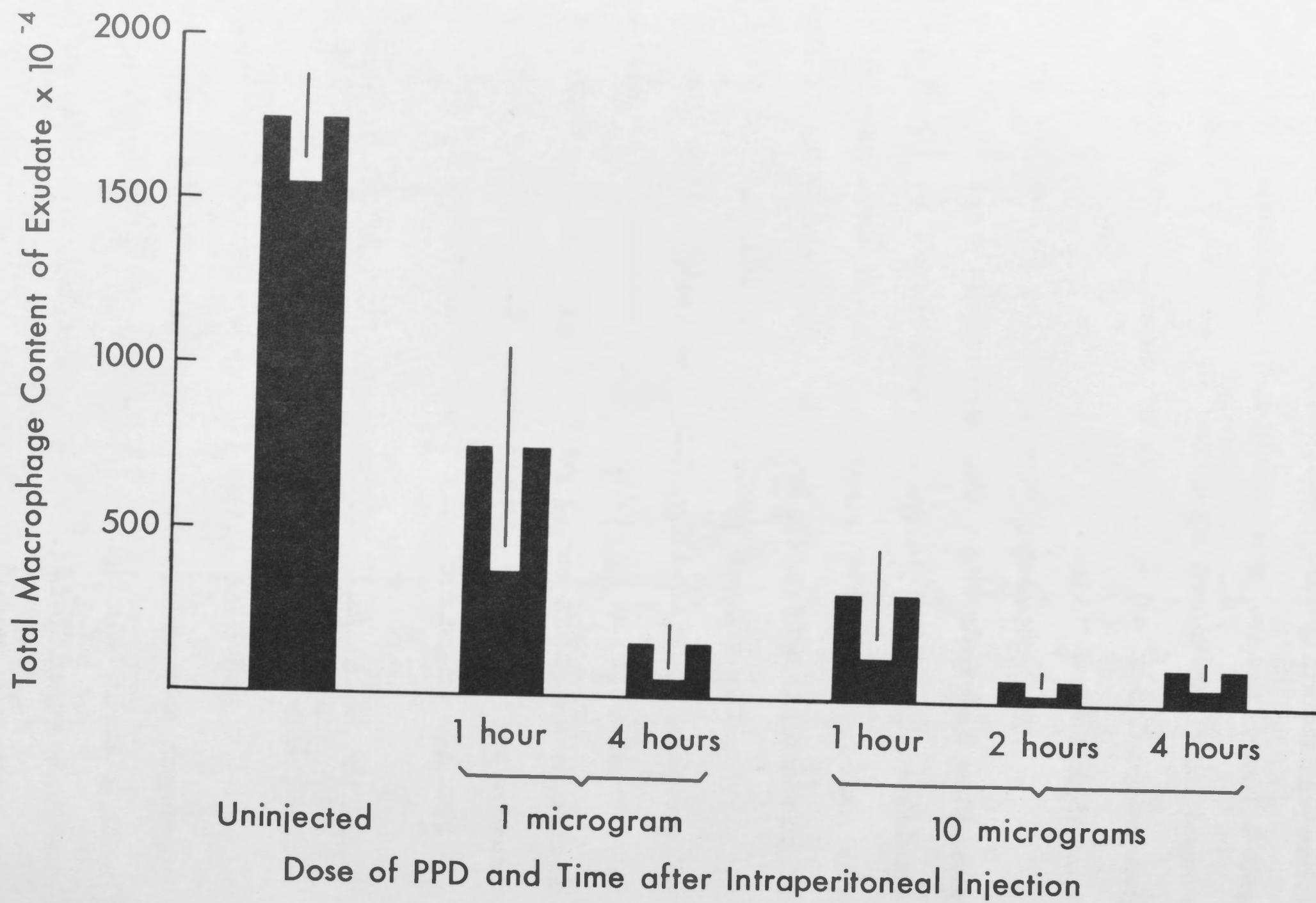
In hypersensitive animals, all doses of PPD caused a depression in the macrophage content of the exudates which was roughly proportional to the dose used. The macrophages of exudates of normal animals were not significantly affected even by 100 micrograms of PPD.

The polymorph content of exudates of both groups of animals was significantly increased after the injection of 100 micrograms of PPD. The lymphocyte counts do not show a consistent trend although the counts for BCG vaccinated animals injected with 0.1 and 100 micrograms of PPD are significantly lower than those of the controls.

The rate of loss of macrophages from peritoneal exudates of guinea pigs with delayed-type hypersensitivity to tuberculin.

BCG-vaccinated (Group 2) guinea pigs with glycogen induced exudates were injected intraperitoneally with 1 or 10 micrograms of PPD 1, 2 or 4 hours before the harvest of exudates. The macrophage contents of these exudates are shown in Figure 2.

Figure 2. The rate of loss of macrophages from peritoneal exudates after intraperitoneal injections of PPD into BCG-vaccinated guinea pigs. The narrow vertical lines indicate the standard error.



It is apparent that the macrophage counts were significantly lowered 1 hour after the injection of either 1 or 10 micrograms of PPD.

The effect of bacterial endotoxin on the cell content of peritoneal exudates.

It has been shown by Packalen and his colleagues (Tunçman and Packalén, 1959; Wasserman and Packalén, 1959) that cells of BCG vaccinated guinea pigs are more susceptible than are those of normal animals to certain non-specific inhibitory substances in vitro (for example, bacterial endotoxin). The possibility that such an effect might be operative in the present series of experiments was investigated.

Normal (Group 1) and BCG-vaccinated (Group 2) guinea pigs were injected intraperitoneally with glycogen and, 4 days later, with varying doses of endotoxin (0.1, 1, 10 and 100 micrograms). The exudates were harvested 4 hours after the latter injection and the cells were counted. Table 6 shows the cell contents of exudates of animals injected with 1 microgram of endotoxin compared with those injected with Hanks' solution alone.

Both groups (normal and BCG-vaccinated) injected with endotoxin showed significantly lowered macrophage counts, to the extent of 66% in the normal animals and

TABLE 6.

Cell Contents of Peritoneal Exudates of Normal and BCG-Vaccinated Guinea Pigs 4 hours after Intraperitoneal Injection of Endotoxin

Group of Animals, Material Injected, Number in Group	Absolute Cell Count \pm Standard Error $\times 10^{-4}$		
	Macrophages	Lymphocytes	Polymorphs
Group 1 - Normal			
Hanks' Solution, 1 ml	8		
Endotoxin, 1 microgram	9		
	1133 \pm 191	819 \pm 126	2257 \pm 692
	382 \pm 92**	840 \pm 209	4738 \pm 555*
Group 2 - BCG-Vaccinated			
Hanks' Solution, 1 ml	6		
Endotoxin, 1 microgram	5		
	1471 \pm 231	1510 \pm 306	1909 \pm 453
	567 \pm 147*	761 \pm 165	5856 \pm 616**

Statistical significance of differences between cell contents of exudates of animals injected with Hanks' Solution and those injected with endotoxin

** $P \leq 0.01$

* $P = 0.01-0.05$

Others are not significant

62% in the sensitive animals. The degree of increase in the polymorph content of the exudates of the two groups was also similar. A similar parallelism was noted when the two groups of animals were injected with smaller or larger doses of endotoxin. It thus seems highly unlikely that the loss of macrophages from exudates after the injection of tuberculin into guinea pigs with delayed-type hypersensitivity to tuberculin is due to a non-specific hypersensitivity to bacterial products in general.

Cell clumping.

A certain amount of cell clumping was noted in the peritoneal exudates of guinea pigs with delayed-type hypersensitivity injected with antigen. In exudates taken 1 hour after the intraperitoneal injection of antigen these clumps consisted largely of macrophages, while in those taken later (after 4 hours) the clumps consisted almost entirely of polymorphs. It is not considered that cell clumping accounts for the apparent loss of macrophages from these exudates, for two reasons. Firstly, clumped cells were included in both total and differential cell counts. Secondly, there were few or no macrophages in the cell clumps found in exudates 4 hours after the injection of antigen. Nevertheless, an investigation of cell clumping may throw some light

on the mechanism by which macrophages disappear from peritoneal exudates following the injection of antigen.

The results of the experiments described above indicate that the injection of tuberculin (PTD) into BCG-vaccinated guinea pigs, which have been injected intraperitoneally 4 days previously with antigen, brings about the virtual disappearance of macrophages from the peritoneal exudate. This effect is observed whether the tuberculin is injected subcutaneously, subcutaneously or intraperitoneally; however, considerably larger amounts of tuberculin are required if the subcutaneous and intraperitoneal routes are used. The macrophages have almost disappeared 1 hour after the intraperitoneal injection of tuberculin; they disappear between 2 and 6 hours after subcutaneous injection.

Several facts strongly suggest that the disappearance of macrophages from the peritoneal cavity is the consequence of a specific immunological reaction. As little as 0.1 microgram of PTD injected intraperitoneally is sufficient to produce this response in BCG-vaccinated guinea pigs, while over 1,000 times this dose (100 micrograms) fails to produce a similar effect in normal animals. The specificity of the reaction is revealed in the experiments in which guinea pigs subcutaneously injected with tuberculin and guinea pigs

DISCUSSION

The results of the experiments described above indicate that the injection of tuberculin (PPD) into BCG-vaccinated guinea pigs, which have been injected intraperitoneally 4 days previously with glycogen, brings about the virtual disappearance of macrophages from the peritoneal exudate. This effect is observed whether the tuberculin is injected intravenously, subcutaneously or intraperitoneally; however, considerably larger amounts of tuberculin are required if the subcutaneous and intravenous routes are used. The macrophages have almost disappeared 1 hour after the intraperitoneal injection of tuberculin; they disappear between 2 and 6 hours after subcutaneous injection.

Several facts strongly suggest that the disappearance of macrophages from the peritoneal cavity is the consequence of a specific immunological reaction. As little as 0.1 microgram of PPD injected intraperitoneally is sufficient to produce this response in BCG-vaccinated guinea pigs, while even 1,000 times this dose (100 micrograms) fails to produce a similar effect in normal animals. The specificity of the reaction is revealed in the experiments in which ovalbumin and tuberculin were injected into different animals; among

the ovalbumin injected animals low macrophage counts occurred in the group which showed delayed-type hypersensitivity to ovalbumin, but not in the BCG-vaccinated or in the normal animals.

The intraperitoneal injection of E. coli endotoxin was also found to result in a marked loss of macrophages from peritoneal exudates. However, the effect of endotoxin was the same in both normal and BCG-vaccinated guinea pigs and this fact speaks against the possibility that the effect of tuberculin is due merely to a non-specific increased susceptibility of macrophages of BCG-vaccinated animals to an endotoxin-like principle in tuberculin.

The next question arising is whether the immunological reaction, of which the disappearance of macrophages from the peritoneal exudate is a consequence, is related especially to the state of delayed-type hypersensitivity or whether it results from a reaction between tuberculin and classical circulating antibody. The findings support the former interpretation. Low macrophage levels were found only in those groups of animals which showed delayed skin reactions, either mixed with Arthus reactions, as in the guinea pigs immunized with ovalbumin in complete adjuvant, or relatively "pure" as in the BCG-vaccinated animals. The intraperitoneal

injection of antigen into animals with no detectable delayed component to their skin reactions but with strong Arthus hypersensitivity to either tuberculin or ovalbumin did not result in average macrophage counts significantly lower than those of control animals.

Thus the evidence is good that the disappearance of macrophages from peritoneal exudates, which occurs following the injection of tuberculin into BCG-vaccinated guinea pigs, is the consequence of an immunological reaction and that this reaction is related to the state of delayed-type hypersensitivity rather than to the presence of circulating antibodies and Arthus hypersensitivity. This conclusion increases interest in the reaction, since an analysis of its mechanism might throw light on the nature of the tuberculin reaction as well as of other states of delayed-type hypersensitivity.

With regard to the mechanism of the reaction two questions are outstanding. Firstly, what is the fate of the macrophages? Have they become firmly adherent to the lining of the peritoneal cavity, have they migrated into the blood stream or lymphatics or have they disappeared as the result of lysis? The other question is whether the macrophages are themselves specifically sensitive to the antigen, that is, whether the antigen acts directly on these cells or whether they are affected

by a product of an immunological reaction involving another type of cell (e.g., the lymphocyte) or perhaps even a specific humoral factor. Further discussion of the possible details of the mechanism of the reaction would hardly be profitable until the answers to these questions are forthcoming. Nor would it seem useful without this information to discuss at length the possible connection between the findings reported above and the rather confusing and often contradictory reports of the effects of tuberculin in vitro on cells from hypersensitive animals. However, certain facts, such as the smallness of the effective dose of tuberculin in sensitive animals, the lack of effect of much larger doses on the macrophage levels of exudates of normal animals and the rapidity with which the macrophage counts drop, suggest that the intraperitoneal reaction may be different from the various in vitro effects of tuberculin which have been described.

SUMMARY

The injection of antigen into guinea pigs with delayed-type hypersensitivity is followed by the prompt disappearance of peritoneal cells. The cells previously induced by tuberculin in the absence of antigen in BCG-vaccinated animals injected with tuberculin was investigated. Clumping of cells prevented their disappearance. Neither clumped nor isolated cells could be stained with trypan blue. The clumps dispersed when incubated *in vitro* in glass chambers; the macrophages adhering to the glass and migrating out of the chambers. Macrophages transplanted in cell-permeable or cell-impermeable millipore chambers into the peritoneal cavities of other guinea pigs remained healthy after the injection of tuberculin into the recipients, even when both donor and recipient were tuberculin sensitive. The examination of histologic smears revealed an increased number of accumulations of cells as well as unclumped cells in hypersensitive animals injected with tuberculin, as compared with controls. Electron micrographs showed that the clumped cells were very closely apposed but did not reveal any notable intercellular material. It is suggested that macrophages of animals

PAPER IV.

THE FATE OF PERITONEAL MACROPHAGES AFTER THE INJECTION OF ANTIGEN INTO GUINEA PIGS WITH DELAYED-TYPE HYPERSENSITIVITY

SUMMARY

The injection of antigen into guinea pigs with delayed-type hypersensitivity is followed by the prompt disappearance of free-floating macrophages from previously induced peritoneal exudates. The fate of these cells in BCG-vaccinated animals injected with tuberculin was investigated. Clumping of cells preceded their disappearance. Neither clumped nor unclumped cells could be stained with trypan blue. The clumps dispersed when incubated in vitro in glass chambers, the macrophages adhering to the glass and migrating out of the clumps. Macrophages transplanted in cell-permeable or cell-impermeable Millipore chambers into the peritoneal cavities of other guinea pigs remained healthy after the injection of tuberculin into the recipients, even when both donor and recipient were tuberculin sensitive. The examination of mesenteric spreads revealed an increased number of accumulations of clumped macrophages as well as unclumped cells in hypersensitive animals injected with tuberculin, as compared with controls. Electron micrographs showed that the clumped cells were very closely apposed but did not reveal any notable intercellular material. It is suggested that macrophages of animals

with delayed-type hypersensitivity become more adhesive in the presence of antigen and that this increased adhesiveness may account for their clumping and disappearance from peritoneal exudates as well as for some other manifestations of delayed-type hypersensitivity.

INTRODUCTION

A state of delayed-type hypersensitivity can accompany or follow many natural or experimental immunological events in animals. These include infection, e.g., with tubercle bacilli; the rejection of allogeneic homografts (Brent, Brown and Medawar, 1962); the injection of foreign protein in adjuvants containing Mycobacteria (Nelson and Boyden, 1963 a); and "auto-immunization" (McMaster, Lerner and Exum, 1961; Flax, Jankovic and Sell, 1963). The most notable feature of the delayed skin reactions which can be elicited in all these conditions is the progressive accumulation of macrophages and other mononuclear cells at the site of injection of the antigen. Epithelial necrosis may occur in very severe reactions but the mononuclear cells usually remain healthy. In contrast, many studies have suggested that antigens may have a toxic effect in vitro on macrophages of animals with delayed-type hypersensitivity (see reviews by Raffel, 1954; Favour, 1957; Boyden, 1958; Waksman, 1958). Recently, it was found that the injection of antigen into guinea pigs with delayed-type hypersensitivity caused the disappearance, preceded by clumping, of macrophages from previously induced peritoneal exudates (Nelson and Boyden, 1963 b). The effect was

observed after intravenous, subcutaneous or intraperitoneal injections of antigens. It was apparent within an hour of the intraperitoneal injection of quite small doses of antigen. The smallness of the effective dose, the rapidity of the reaction and the absence of obvious cell debris in the peritoneal washings all suggested that this was not a toxic effect of the antigen on the cells. Further investigations of the nature of this reaction and the fate of the cells are reported here.

Sensitization. Guinea pigs were injected intradermally with 0.1 mg. BCG (Commonwealth Serum Laboratories, Melbourne) 4-6 weeks before use.

The Purified Protein Derivative (PPD) of tuberculin (supplied by the State Serum Institute, Copenhagen, Denmark) was used in all experiments. Batches RT 22 and Batch RT 23, of comparable potency, were used.

Estimation of cell viability. A 1% solution of Trypan Blue in normal saline was prepared. This was filtered immediately before use and 0.3 ml. was added to 1 ml. of the fluid harvested from the peritoneal cavity. The mixture was incubated in a water bath at 37°C for 30 minutes, then examined microscopically. The staining of the nucleus of cells was considered as indicative of cell death.

MATERIALS AND METHODS

Albino guinea pigs of either sex were used in these experiments. The origin and care of these animals have been described previously, as have the methods of induction of peritoneal exudates by glycogen, of harvesting the exudates and of counting the cells therein (Nelson and Boyden, 1963 b).

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Cell culture in vitro. The slide chambers used have been described in detail by Vaughan and Boyden (1963 a). Thick perspex slides had holes 1 cm. in diameter bored in the centre. One side was covered by a coverslip fixed to the slide by paraffin. The shallow well so formed was filled with the cell suspension, containing $0.5-2.0 \times 10^6$ cells/ml., then sealed with a second coverslip. The slide was incubated at 37°C for 10 minutes by which time the macrophages in the cell suspension had adhered to the lower coverslip. The slide was then inverted and the attached cells observed by means of phase contrast microscopy at 37°C . The composition of the medium used varied, but consisted basically of Hanks' balanced salt solution containing penicillin (50 units/ml.) and streptomycin (100 units/ml.) (HPS) to which were added heparin, guinea pig serum (Lyophilized Complement, Commonwealth Serum Laboratories, Melbourne) or PPD, as described below.

Millipore chambers. Millipore filters (Millipore Filter Corp., Bedford, Mass.) 1 cm. in diameter, were attached to perspex rings by means of Millipore cement. The lower side of the ring was covered with a filter of pore size 0.3 microns. The pore size of the filter covering the other side was either 0.3 microns (cell-impermeable) or

5 microns (cell-permeable). In some experiments perforations 1-2 mm. in diameter were made in one filter. The perspex rings were 3 mm. in thickness and each had two holes bored radially at right angles, through one of which the cell suspension was injected. After the chambers were filled, these holes were sealed with paraffin and the chambers were incubated at 37°C for 10 min. in order to allow the cells to settle on and adhere to the lower filter. The chambers held 0.2-0.3 ml. of fluid containing $0.5-2.0 \times 10^6$ cells/ml. Recipient guinea pigs were anaesthetized with ether and the chambers were placed in their peritoneal cavities through a small midline incision in the previously shaved abdomen. The incision was closed with silk sutures and covered with tulle gras, a layer of gauze bandage and a further layer of Elastoplast wound around the body. After the chambers were removed from the recipients, the filters were cut out with a scalpel, washed in Hanks' solution, fixed in Bouin's solution and stained with Mayer's Haemalum and Congo Red (Vaughan and Boyden, 1963 b).

Mesenteric spreads. After the peritoneal cavities had been washed out, 2 or 3 pieces of mesentery with the gut still attached were excised and spread out on a microscope slide. They were usually of a size sufficient

to cover about half the area of the slide. They were immediately fixed in methanol for 10 min. washed with distilled water and stained for 20 min. with Giemsa stain diluted 1:5 in distilled water. They were then washed again in distilled water and allowed to dry, after which the gut was cut off with scissors.

Electron microscopy. A portion of the peritoneal fluid was centrifuged and the supernatant was removed. The pellet of cells was teased away from the tube and immersed in a balanced salt solution containing 1% osmium tetroxide and buffered according to Palade (1952). The cells were then washed in several changes of 1% uranyl acetate and allowed to remain in the final solution for 1 hour.

Dehydration was carried out with 70% and 100% ethanol.

Araldite was used for embedding. Sections were cut on a Porter-Blum microtome with a diamond knife and collected onto carbon films supported by copper specimen grids.

Micrographs were taken with a Siemens Elmiskop I electron microscope at an instrumental magnification of 30,000 times.

RESULTSLACK OF CYTOTOXIC EFFECT OF TUBERCULIN IN VIVO

It had previously been found that intraperitoneal injections of PPD into BCG-vaccinated guinea pigs caused the virtual disappearance of macrophages from peritoneal exudates within 1-2 hours. It had also been noted that the macrophages were clumped, but had not disappeared 20-30 min. after the injection of PPD (Nelson and Boyden, 1963 b and unpublished experiments). The purpose of the experiment described here was to determine whether the macrophages were dead when they formed clumps.

Normal and BCG-vaccinated guinea pigs were injected intraperitoneally with glycogen. Four days later some of these animals were injected intraperitoneally with PPD (10 or 100 micrograms) and were sacrificed 20 or 30 min. later, when the exudates were harvested. Exudates were also harvested from control animals which had received no PPD. The degree of cell clumping was noted and the proportion of cells which were dead was estimated by staining with Trypan Blue. To ensure that the staining procedure detected cell death, an aliquot of an exudate from a normal animal, not injected with PPD, was heated to 56°C for 1 hr. before incubation with Trypan Blue.

TABLE 1.

Lack of Cytotoxicity of Tuberculin in vivo

Guinea Pigs	Dose of PPD micrograms	Time after injection, minutes	Cell clumps	Percentage of cells stained with Trypan Blue
Normal	0	-	-	0
Normal (killed cells) ^a	0	-	-	100
Normal	10	20	-	0
"	10	30	-	1
"	100	20	-	0
"	100	30	-	0
BCG- vaccinated	0	-	-	0
"	0	-	-	1
BCG- vaccinated	10	20	+	0
"	10	20	++	1
"	10	30	+	1
"	10	30	++	0
"	100	20	++	1
"	100	20	++	1

^a Cells killed by heat (56°C for 1 hour)

The results are shown in Table 1.

The heat-killed cells all showed nuclear staining with Trypan Blue. There was no cell clumping in the exudates from normal animals injected or not injected with PPD or in the exudates of BCG-vaccinated animals not injected with PPD. The proportion of cells in these exudates stained with Trypan Blue did not exceed 1%. There was marked cell clumping in the exudates of the BCG-vaccinated animals injected with PPD. The clumps contained between 10 and 50 cells, all of which appeared to be macrophages. Again, the proportion of cells stained with Trypan Blue did not exceed 1%. It was noted that even the cells in clumps were not stained. These results indicate that cell death is probably not a factor in the cell clumping which precedes the disappearance of macrophages from peritoneal exudates of hypersensitive guinea pigs injected with antigen.

REVERSIBILITY OF CELL CLUMPING IN VITRO

These experiments were carried out to determine whether the macrophages of hypersensitive animals injected with antigen would die after clumping, that is, whether clumping was a reflection of damage short of actual cell

TABLE 2.

Media with which Peritoneal Cells were
Incubated in vitro

Cells from peritoneal exudates	Medium			
	HPS	Normal guinea pig serum	Heparin	PPD
A. As removed from peritoneal cavity	+	3%	5 units/ ml.	+ ^a
B. Centrifuged and resuspended in medium	+	20%	5 units/ ml.	-
	+	20%	-	-
	+	20%	-	10 micro- grams/ ml.
	+	20%	-	10 micro- grams/ ml.

^a PPD (10 micrograms) injected intraperitoneally:
20-30 min. before harvest.

death. Because most of the macrophages disappeared from the peritoneal exudates shortly after clumping in vivo, it was necessary to remove them from the peritoneal cavity and incubate them in vitro.

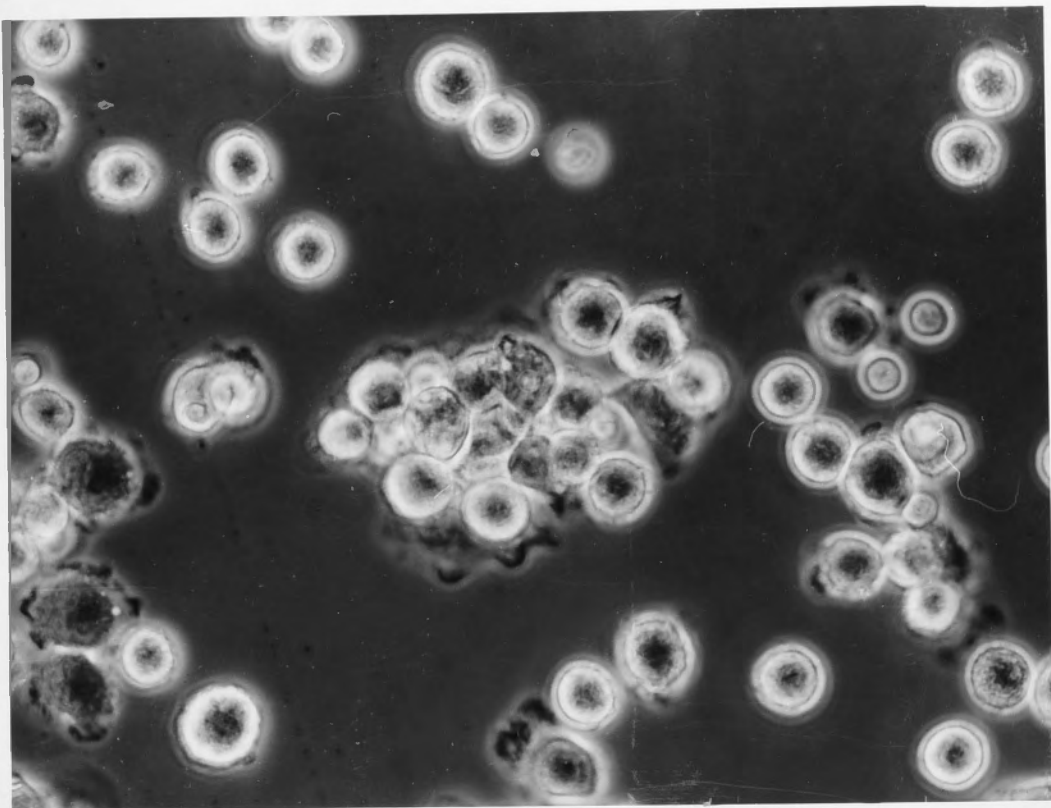
Exudates were induced in normal and BCG-vaccinated guinea pigs by intraperitoneal injections of glycogen. After four days PPD (10 micrograms) was injected intraperitoneally and the exudates were harvested 20-30 mins. later. The cells were introduced into slide chambers. The media in which the cells were incubated are shown in Table 2.

As in the previous experiment clumping of macrophages occurred only in BCG-vaccinated guinea pigs injected with PPD. Incubation for 10 min. at 37°C sufficed to allow both clumped and single macrophages to adhere to the lower coverslip of the chamber. The clumps began to disperse after 15-20 min. of incubation. The macrophages closest to the glass moved slowly out of the clumps, apparently adhering all the time to the glass. Other cells which had not clumped in vivo were also motile, so that the picture changed slowly all the time. A short broad process or processes marked the anterior end of the cell, while there were longer and narrower processes at the posterior end. The clumps had usually dispersed after

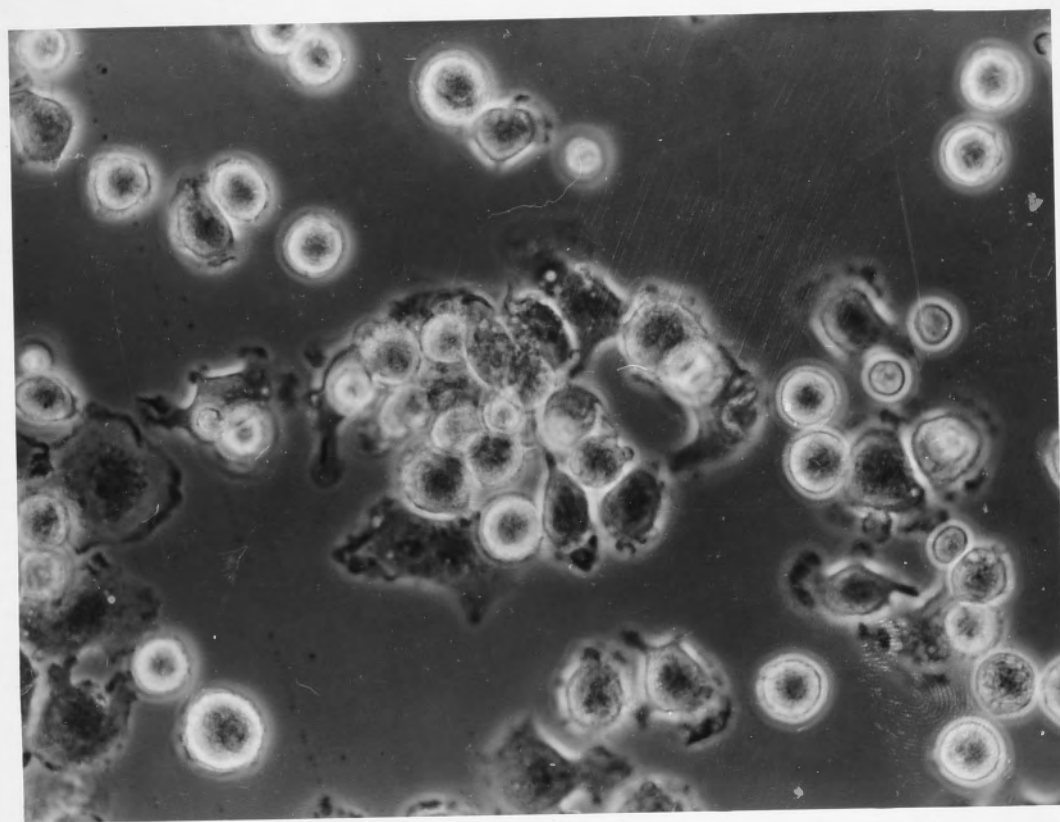
Figure 1. Dispersal in vitro of clumped macrophages.

Photomicrographs of the same field after (A) 10 min. incubation, (B) 15 min. incubation, (C) 40 min. incubation in vitro in slide chambers at 37°C. The cells were harvested from a BCG-vaccinated guinea pig 20 min. after it had received 100 micrograms PPD i.p. (Phase contrast, x 1920).

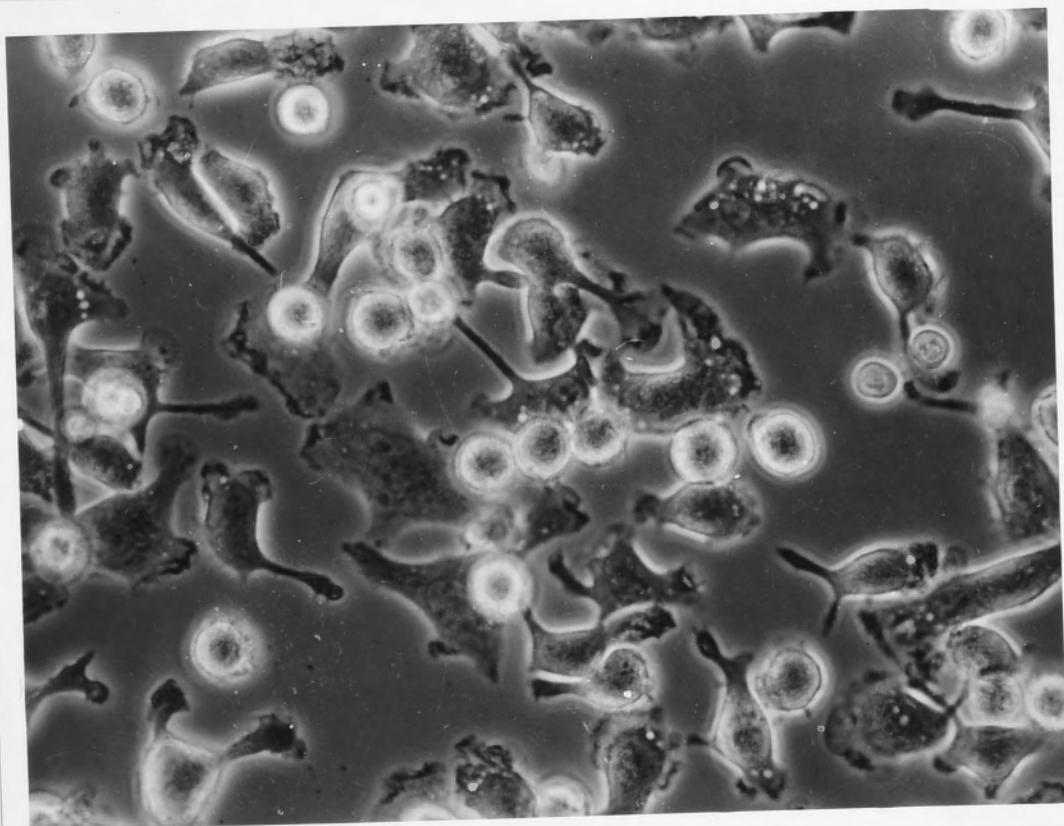
A



B



C



30-60 min. in cultures observed continuously at 37°C. Figure 1 illustrates the dispersal of a clump of macrophages. The composition of the medium did not affect the occurrence or rate of dispersal, which was as rapid in media containing PPD as in media containing no PPD. The presence of heparin in the medium did not affect either the adherence of cells to glass or the rate of dispersal of the clumps.

These experiments further support the idea that the disappearance of macrophages is not a consequence of cell damage or cell death.

INTRAPERITONEAL TRANSPLANTATION OF MACROPHAGES IN MILLIPORE CHAMBERS

These experiments were carried out to determine whether or not transplanted macrophages were affected by antigen in vivo. Because macrophages transplanted from one animal to another usually become dispersed throughout the body (Mims, 1963) the cells were enclosed in Millipore chambers.

Samples of peritoneal washings from both normal and BCG-vaccinated guinea pigs injected 4 days previously with glycogen were introduced into Millipore chambers which were then placed in the peritoneal cavities of both normal

TABLE 3.

Plan of Experiments using Cells Transplanted
in Millipore Chambers

Cell donor	Recipient	Dose of PPD injected into recipient
1. Normal	Normal	100 micrograms
2. Normal	Normal	0
3. BCG-vaccinated	Normal	100 micrograms
4. BCG-vaccinated	Normal	0
5. Normal	BCG-vaccinated	100 micrograms
6. Normal	BCG-vaccinated	0
7. BCG-vaccinated	BCG-vaccinated	100 micrograms
8. BCG-vaccinated	BCG-vaccinated	0

and BCG-vaccinated guinea pigs injected 2 days previously with glycogen. The recipients were sacrificed after a further 2 days. Some of the recipients had been injected intraperitoneally with 100 micrograms PPD 4 hrs. before sacrifice. The plan of the experiments is shown in Table 3. At sacrifice, the Millipore chambers were removed and the peritoneal exudates of BCG-vaccinated animals injected with PPD were harvested.

When the animals were sacrificed the chambers were frequently found to be surrounded by mesentery, omentum or fibrin. Microscopic examination of the stained filters showed that the outer surfaces were covered with polymorphs and macrophages. Surprisingly, there was little or no evidence of migration of host cells through the filters of pore size 5 microns, which had been presumed to be cell-permeable. The donor cells were adherent to the inner side of the filter onto which they had been allowed to settle before the chambers were inserted. These donor cells were almost entirely macrophages. In all cases they appeared healthy, being well spread out on the surface of the filter and frequently having pseudopodia¹. No differences were

¹Although these preparations were quite suitable for microscopic examination the density of the filters together with the attached cells made them unsuitable for photography.

observed in the numbers and morphology of the donor macrophages among the different groups. The macrophages disappeared almost completely from the peritoneal exudates of BCG-vaccinated recipients injected with PPD. When large perforations had been made in the filter on the upper side of the chamber to allow the freest possible ingress and egress of cells, the donor cells on the lower filter were unaffected in either numbers or morphology. When BCG-vaccinated animals carrying such chambers filled with cells from BCG-vaccinated donors were injected with PPD, most of the donor cells appeared to be unaffected, although occasionally aggregations of macrophages were seen on the filter. These were presumed to be of host origin, but some of them may have been attached to donor cells.

Therefore This experiment showed that macrophages transplanted in Millipore chambers were not damaged after intraperitoneal injections of PPD, even when both donor and recipient were tuberculin-sensitive.

Exudates were induced by intraperitoneal injections of glycogen 4 days before the animals were used. Some of the animals

EXAMINATION OF MESENTERIC SPREADS

subcutaneous All of the experiments described indicated that cell death could not account for the loss of macrophages exudates had been washed out in the usual way.

from the peritoneal exudates of hypersensitive guinea pigs injected with antigen. These findings, together with the occurrence of cell clumping, suggested that the change induced in these cells by antigen might be an increased stickiness which could cause the cells to adhere ~~not only~~ ^{here} to each other but also to the lining of the peritoneal cavity. It was hoped that examination of the mesenteries of experimental and control animals might reveal the fate of the macrophages.

Initially, mesenteric spreads were prepared from animals which had received intraperitoneal injections of PPD some 2 to 4 hours previously. The marked polymorphonuclear exudation in both normal and BCG-vaccinated animals obscured the histological picture so much that no conclusions could be drawn. Advantage was therefore taken of the fact that subcutaneous injections of PPD also cause the disappearance of macrophages from peritoneal exudates in hypersensitive animals (Nelson and Boyden, 1963 b).

Exudates were induced by intraperitoneal injections of glycogen 4 days before the animals were used. Some of the animals received 400 micrograms PPD injected subcutaneously into the groin 4, 5 or 6 hours before sacrifice. Mesenteric spreads were prepared after the exudates had been washed out in the usual way.

Macrophages were seen on the mesenteries in all animals. In the control guinea pigs they were seen in the following situations:

- (1) Scattered evenly and singly over the whole mesentery (Figure 2).
- (2) Associated with very large aggregations of cells several mm. wide and many cells thick (Figure 3). These were rare. Most of the cells in these aggregations were fibroblasts or serosal cells.
- (3) In small dense clumps of 5 to 20 cells irregularly scattered over the mesentery (Figure 2).

In the BCG-vaccinated guinea pigs injected with PPD macrophages were seen in the same situations (1) and (2) as in control animals. Macrophages were also seen in these animals in the following situations:

- (3) In small dense clumps of 10-50 cells irregularly scattered over the mesentery (Figure 4). These clumps were larger and much more numerous than in control animals.
- (4) As single cells or small loose clumps of cells in close relation to the blood vessels (Figures 5 and 6). These also were very common.

Figure 2. A and B.

Mesenteric spreads from control guinea pigs.

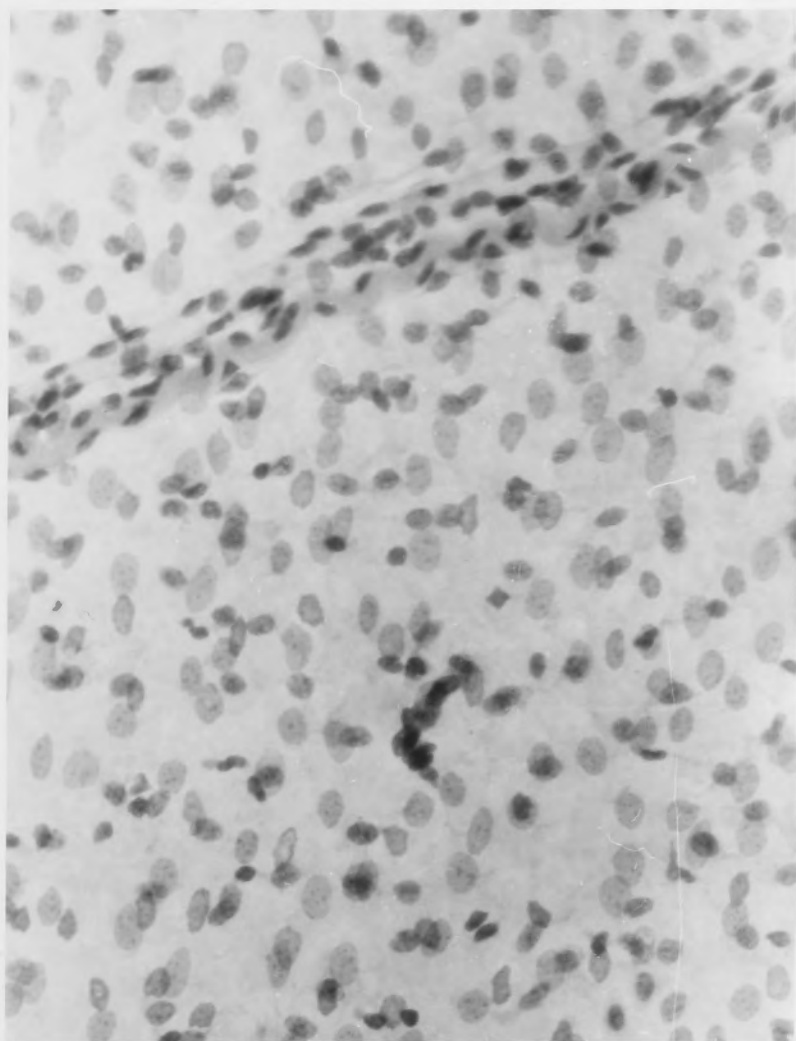
- A. A leash of vessels is shown. The nuclei of the serosal cells are large and pale; those of the macrophages are smaller and darker. There is a very small clump of macrophages.
- B. Similar to A, but showing a larger clump of macrophages.

Figure 3.

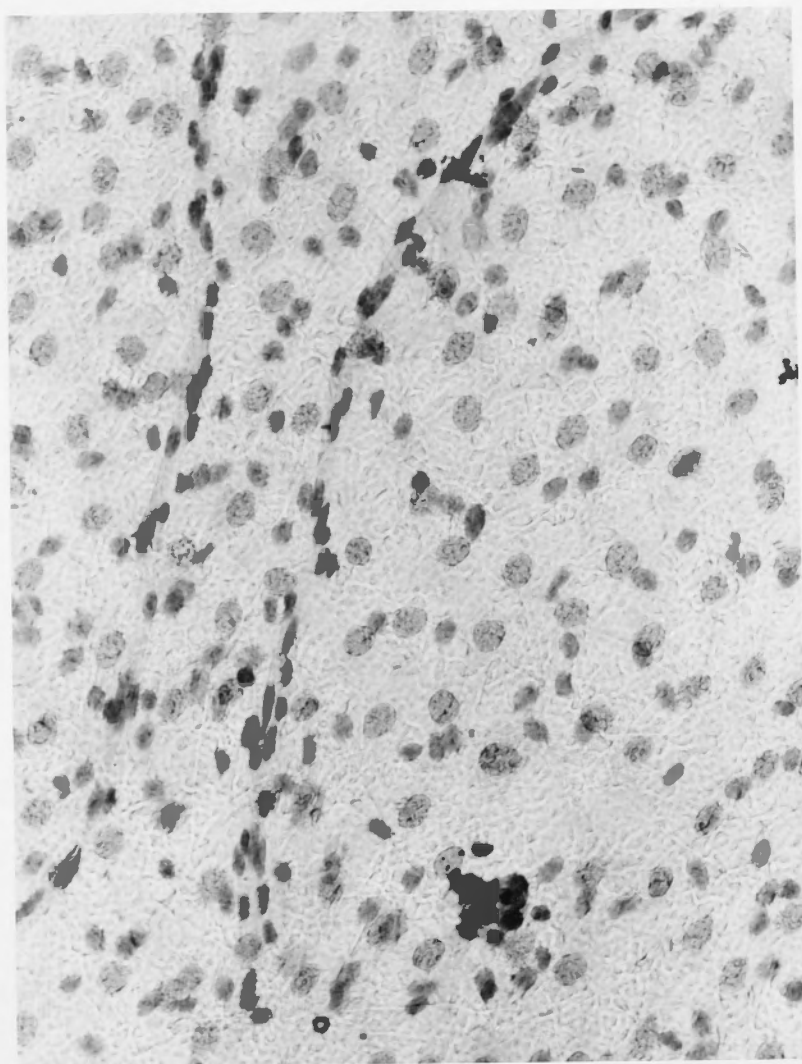
Mesenteric spread from control guinea pig.

This photomicrograph shows a dense aggregation of cells (?fibroblasts) with which is associated a small clump of macrophages (small dense nuclei on right).

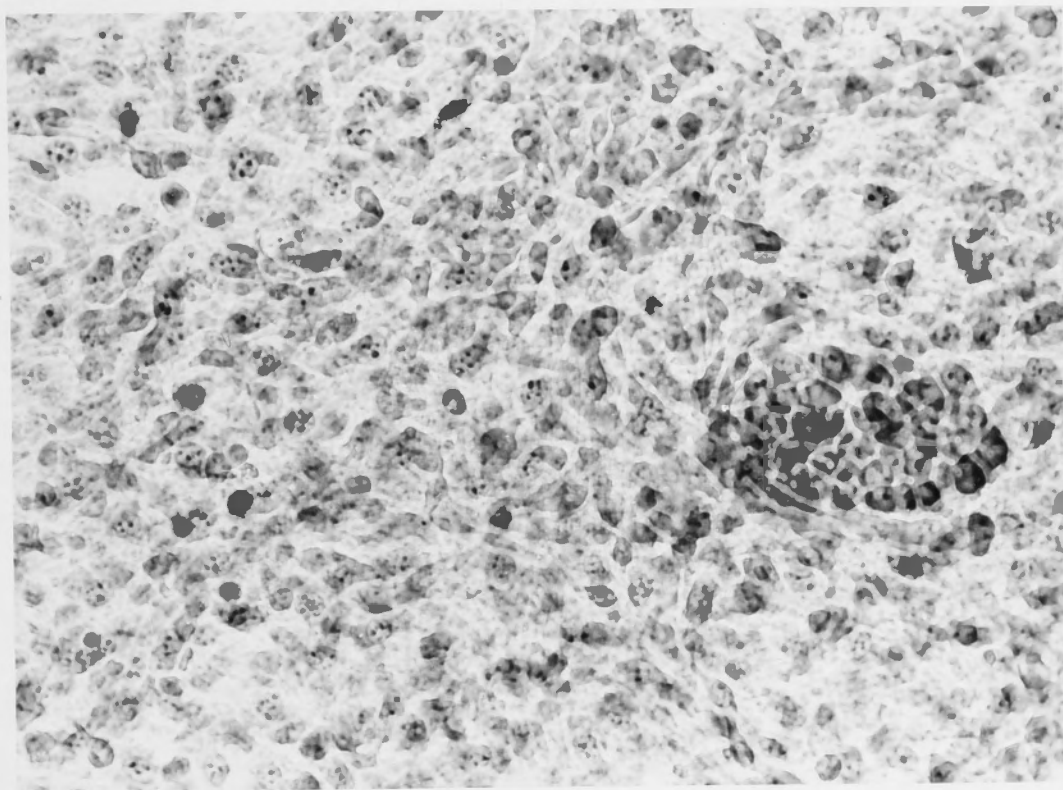
(All stained with Giemsa; x 512).



2A



2B



3

Figures 4, 5 and 6.

Mesenteric spreads from BCG-vaccinated guinea pigs injected subcutaneously with PPD (400 micrograms) 4 to 6 hours previously.

Figure 4.

Large, dense clumps of macrophages.

Figure 5.

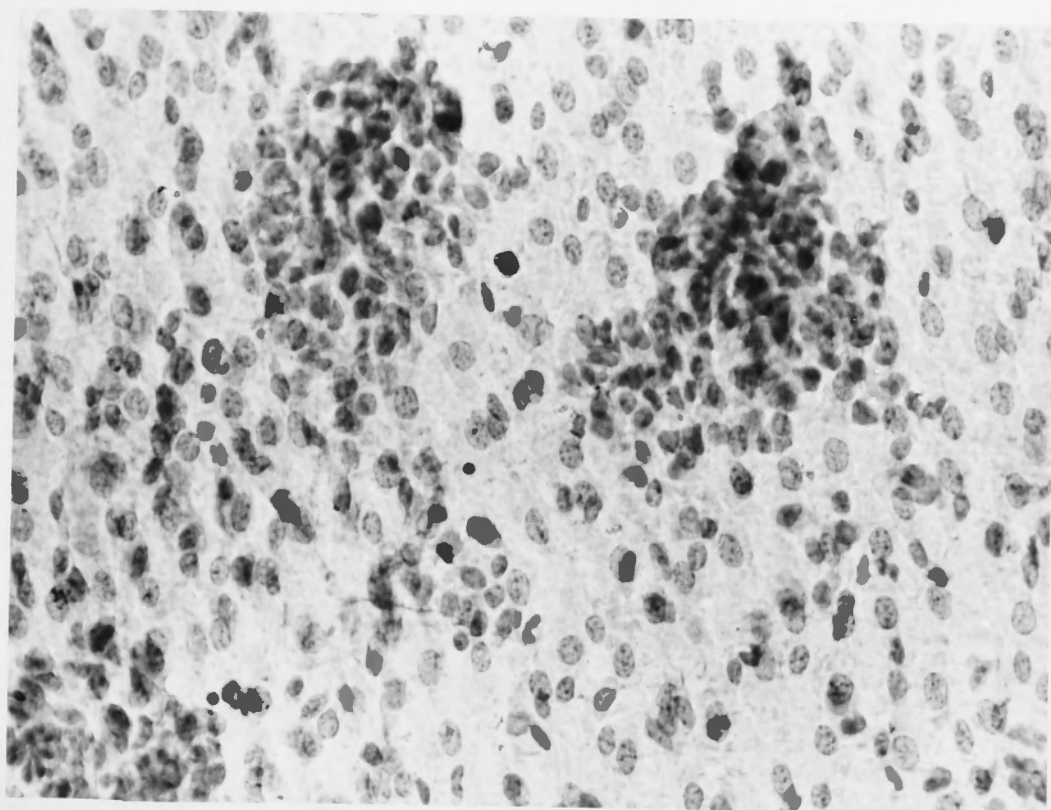
Loosely clumped macrophages adherent to the mesentery around a blood vessel.

Figure 6.

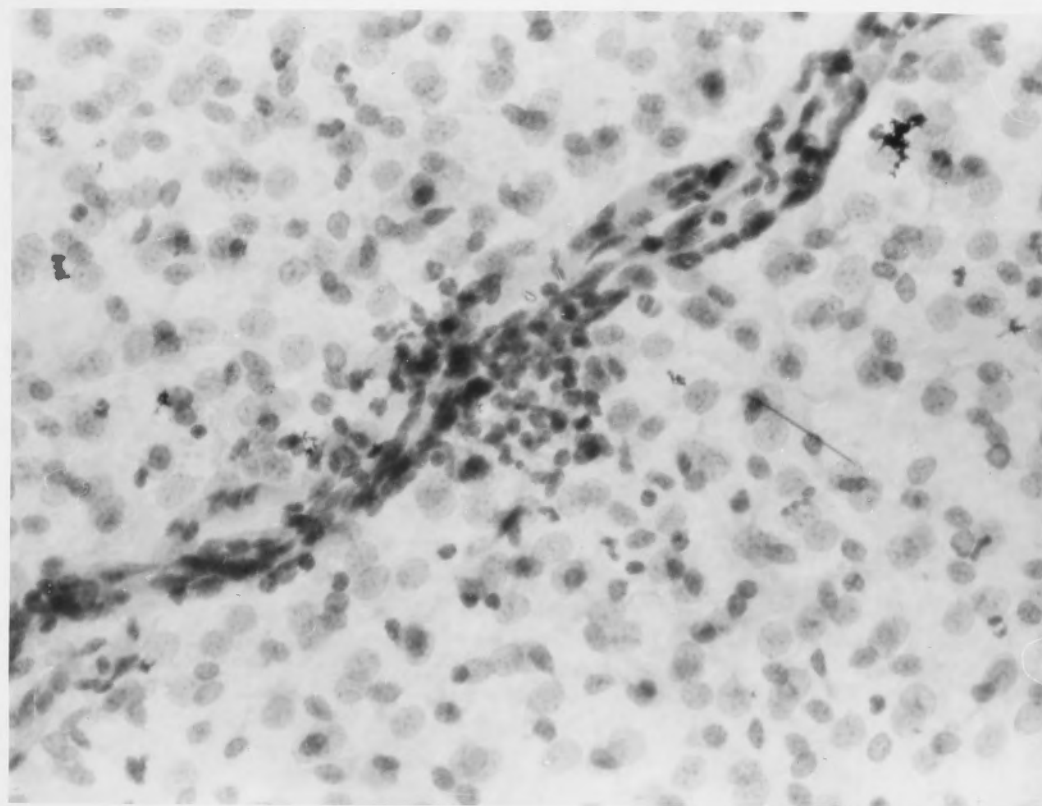
Many single macrophages adherent to mesentery near a blood vessel.

(All stained with Giemsa; x 512 approx.).

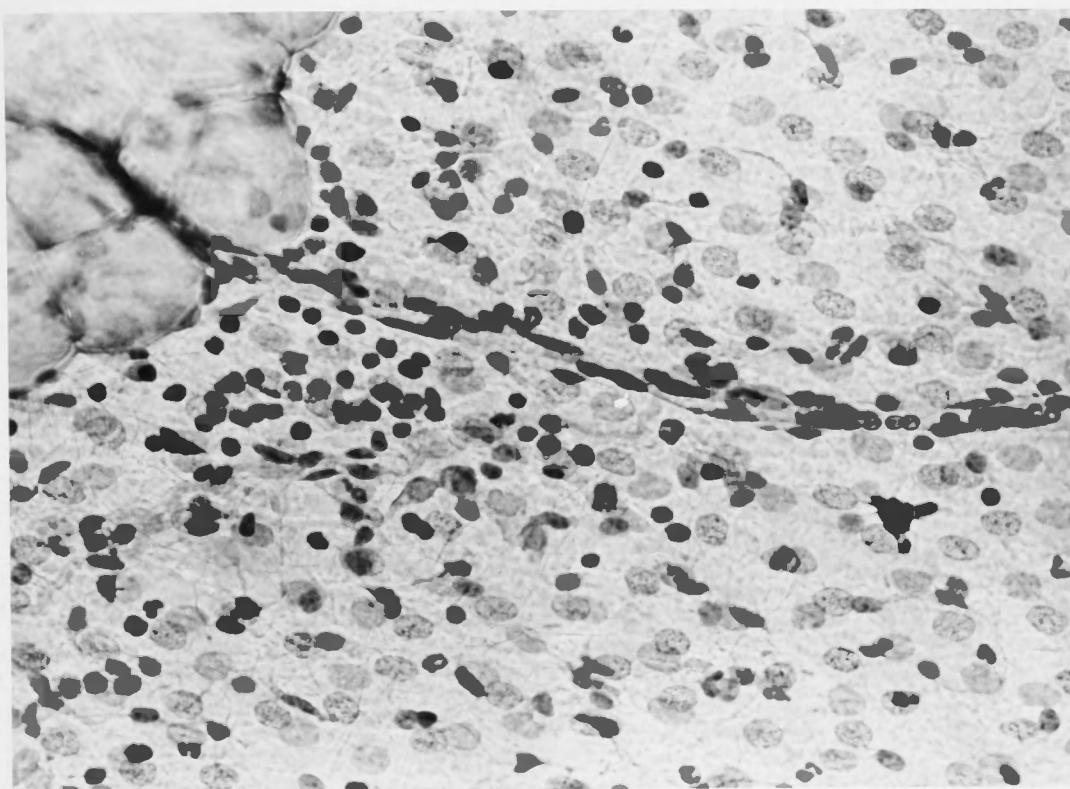
4



5



6



These findings strongly suggest that the loss of macrophages from the peritoneal exudates might be accounted for by their adherence to the lining of the peritoneal cavity singly or in clumps.

ELECTRONMICROSCOPIC EXAMINATION OF CLUMPED CELLS

It seemed likely that the peritoneal macrophages of guinea pigs with delayed-type hypersensitivity reacted to antigen by adhering to each other and to the lining of the peritoneal cavity. It was thought possible that some material between the cells or some change in the surface ultrastructure of the cells might be demonstrable.

Peritoneal exudates were induced with glycogen in 2 normal and 2 BCG-vaccinated guinea pigs. Four days later the exudates were harvested, 1 normal animal and 1 BCG-vaccinated animal having received 10 micrograms PPD intraperitoneally 20 minutes before harvest. Samples of each exudate were centrifuged and the pellets were processed for electron microscopy as described above.

The electron micrographs showed that the clumped cells were extremely closely apposed, the distance between the membranes of adjacent cells being as small as 50 Å (Figure 7). In some cases the intertwining of microvilli

Figure 7.

Electron micrograph of clumped macrophages.
Peritoneal macrophages from a BCG-vaccinated
guinea pig injected i.p. with 100 micrograms
PPD 20 min. previously. The cells are in
close apposition. x 45,000.

Inset: Enlargement showing portion of the
membranes of the two cells separated
by a distinct space.

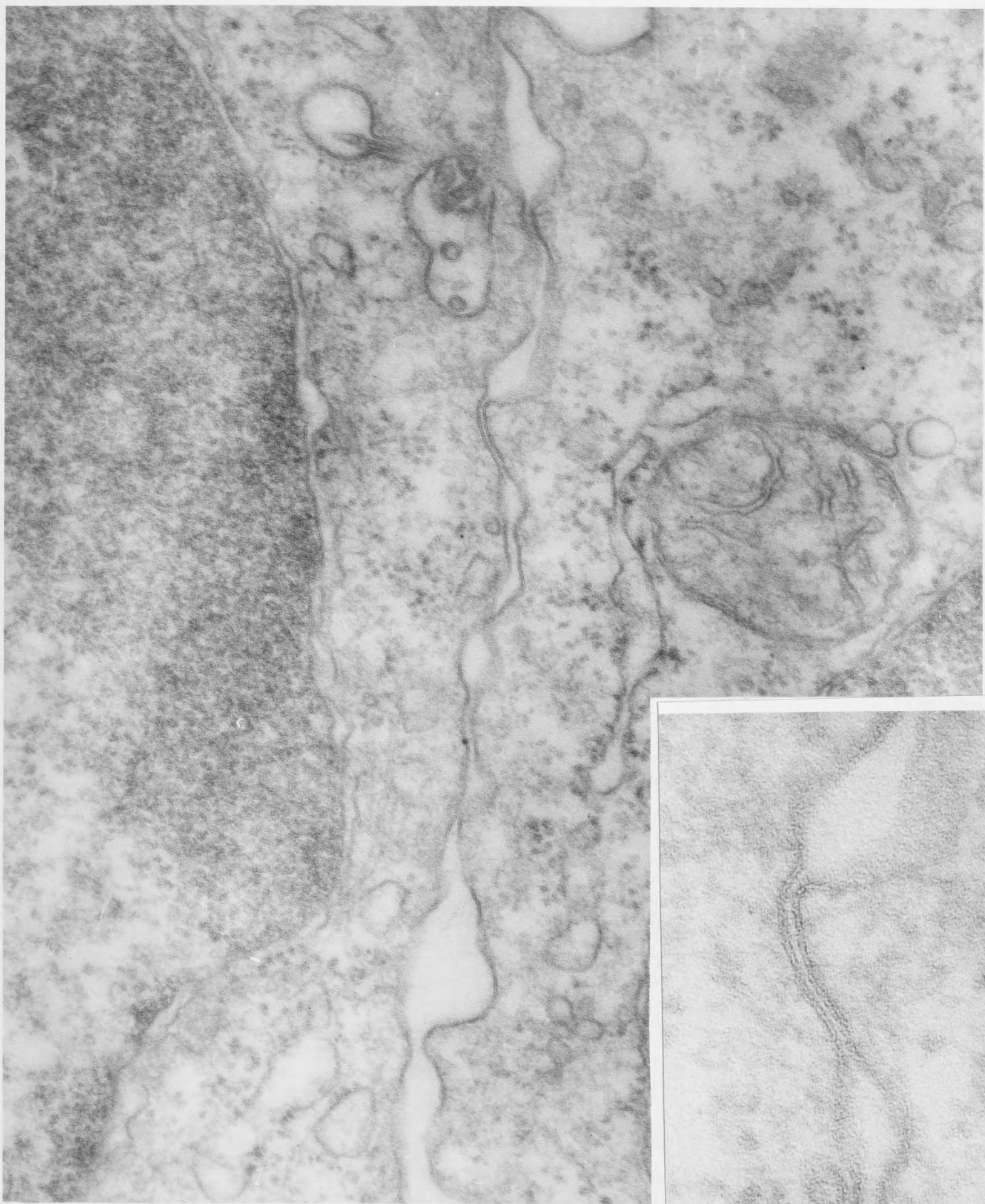
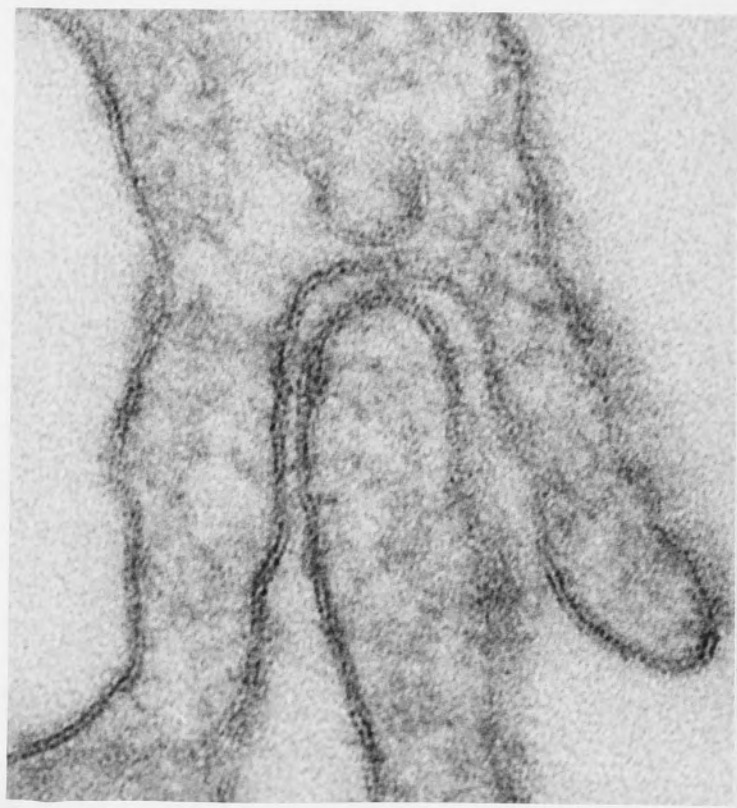
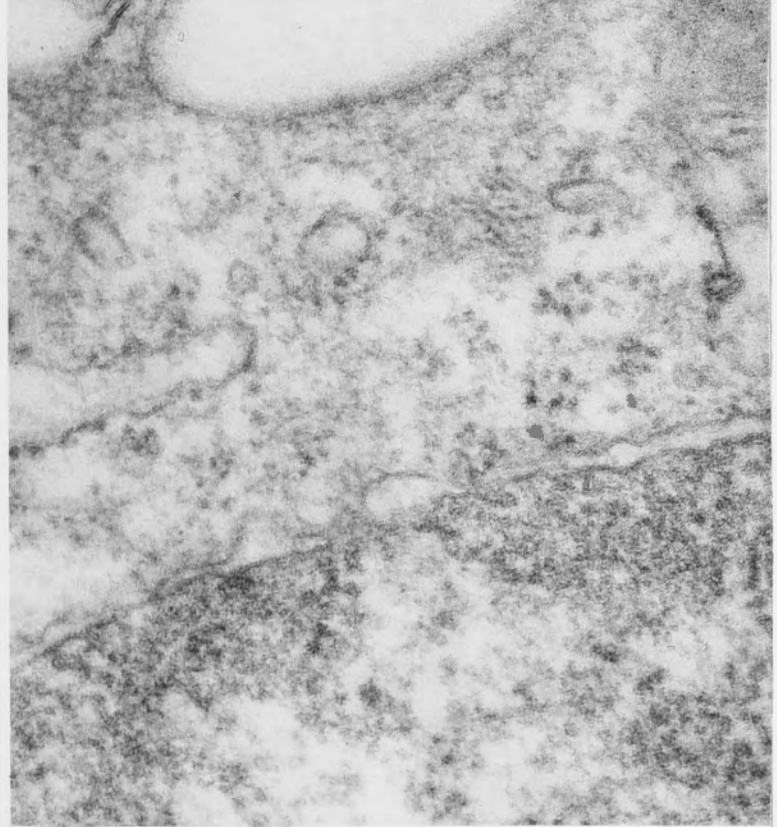
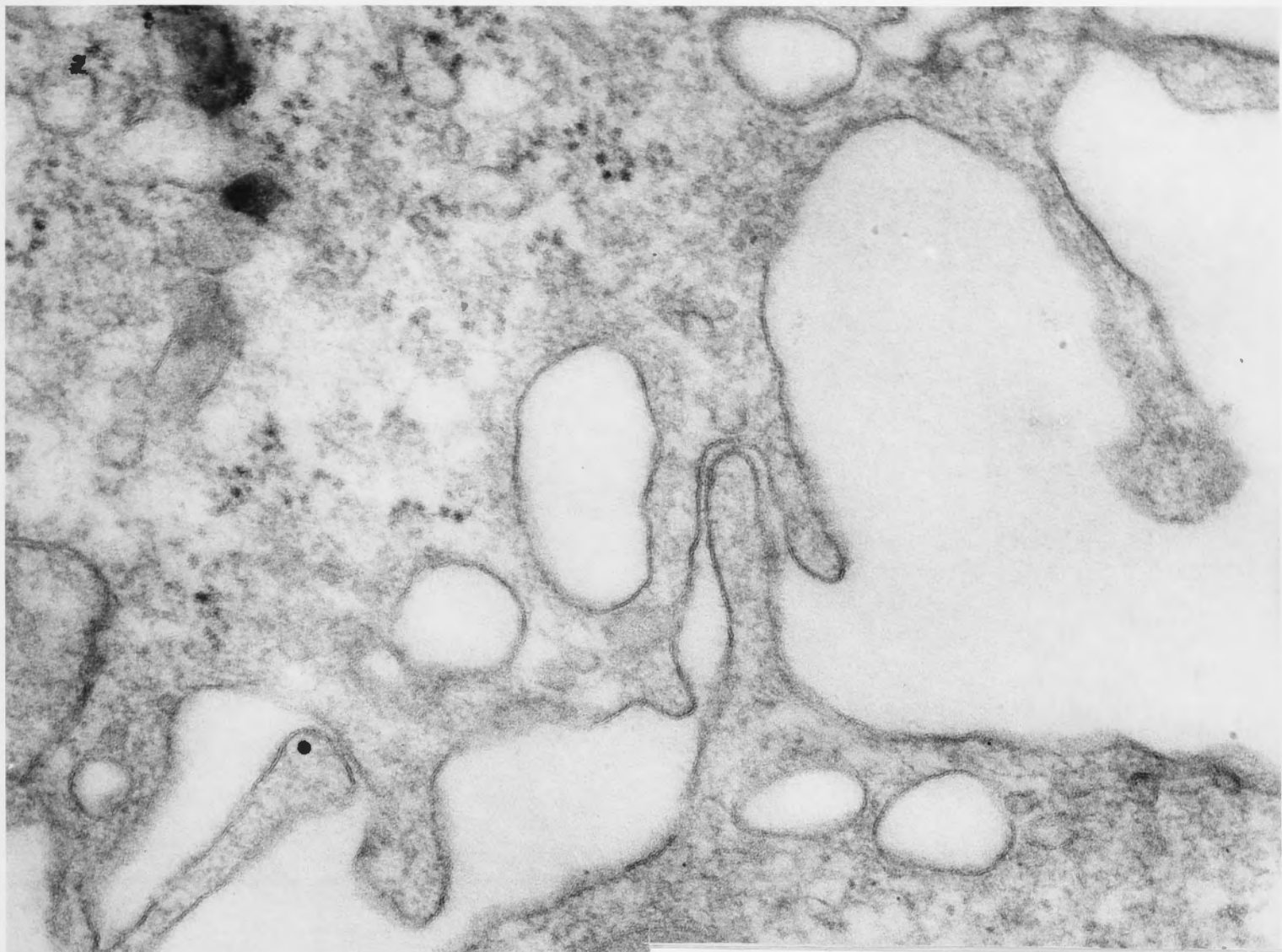


Figure 8.

Electron micrograph of clumped macrophages
Peritoneal macrophages from a BCG-vaccinated
guinea pig injected i.p. with 10 micrograms
PPD 20 min. previously. The cells are in
close apposition and the microvilli are
intertwined. x 45,000.

Inset. Enlargement of microvilli. The space
between the cell membranes is apparent
except where they have not been cut in
true transverse section.



was seen (Figure 8). No material stainable with osmium or uranium was seen between the cells.⁶⁷ It seems therefore unlikely that the attachment of cell to cell could be mediated by fibrin.

DISCUSSION

Cell death does not seem to be a factor in the disappearance of free floating macrophages from the peritoneal exudates after the injection of antigen into guinea pigs with delayed-type hypersensitivity. This view is supported by: the absence of obvious cell debris from the exudates; the failure of cells clumped prior to disappearance to stain with Trypan Blue; the ability of these cells to adhere to glass after their removal from the peritoneal cavity; and the ability of cells in clumps to migrate out of the clumps in vitro. The similar behaviour in short term cell cultures of macrophages from hypersensitive animals injected with antigen and macrophages from control animals also suggests that the cells have not been significantly damaged.

This conclusion is further supported by the absence of any morphological change in macrophages cultured in vivo in Millipore chambers, even after the injection of antigen into a hypersensitive recipient carrying cells from a hypersensitive donor. Doubt may be cast on the value of these experiments because the chambers were frequently surrounded by omentum and/or fibrin clot. Such an objection seems to be negated by the following facts: (1) host cells could still attach themselves to

the outside surfaces of the filters; (2) the cells inside the chambers remained morphologically normal, indicating that the chambers were permeable to nutrient materials; (3) in some experiments with chambers covered by a perforated filter the entry of clumped macrophages was detected following the injection of antigen into hypersensitive hosts.

It seems unlikely that the macrophage disappearance reaction could be due to the migration of cells out of the peritoneal cavity. It is difficult to conceive of cells leaving the cavity in response both to antigen injected intraperitoneally and to antigen injected extraperitoneally and all routes of injection of antigen are equally effective in procuring the disappearance of macrophages (Nelson and Boyden, 1963 b).

The most likely explanation of the disappearance of macrophages is that they adhere, singly or in clumps, to the endothelium lining the peritoneal cavity. The appearance of mesenteric spreads from hypersensitive guinea pigs injected with antigen, compared with those from control animals, lends considerable support to this idea. When macrophages were absent from peritoneal washings after subcutaneous injections of PPD into BCG-vaccinated guinea pigs, there were very commonly large

clumps of these cells attached to the mesenteries as well as smaller clumps and single cells closely related to small blood vessels. Such clumps and perivascular accumulations of cells were seen but rarely in mesenteric spreads from control animals. In mice such accumulations are much more common than in guinea pigs but Mims (1963) has noted that they are even more numerous in BCG-vaccinated mice after the intravenous injection of PPD.

Both the occurrence of cell clumping and the attachment of cells to the endothelium of the peritoneal cavity suggest that the change in the macrophages of hypersensitive animals brought about by antigen is an increased stickiness, resulting, in these experiments, in their adherence to each other and to the peritoneal endothelium. Such a change could also bring about the attachment of these cells to a foreign surface, as in the experiments in which clumped macrophages were seen to be attached to the surface of Millipore filters. The lack of morphological change in the cells on Millipore filters after antigen injection, even when both donor and recipient were hypersensitive, is compatible with this conclusion. In this case, the cells being firmly adherent to a foreign surface, no further evidence of increased stickiness could be expected.

The cause of the increased stickiness is unknown.

The "macrophage disappearance reaction" is inhibited by anticoagulants, which suggests that some factor associated with blood coagulation may be involved (Nelson, 1963).

The electron microscopic studies described here indicate, however, that the "sticky substance" is probably not fibrin. It may be that the increased stickiness is a reflection simply of general metabolic stimulation of macrophages, a normal property of these cells being their adhesiveness in many situations. The speed with which clumping occurs makes this hypothesis rather dubious.

The experiments described in this paper do not exclude the possibility that the attachment of macrophages to each other and to the lining of the peritoneal cavity is due simply to the formation of complexes between antigen and cytophilic antibody attached to the cells (Boyden, 1963). Other evidence makes this hypothesis unlikely. The macrophage disappearance reaction is completely inhibited in guinea pigs pretreated with large doses of heparin. The uptake of antigen in vitro by the cells of guinea pigs with delayed-type hypersensitivity is, however, not significantly inhibited by heparin (Nelson and Boyden, 1963 c). Furthermore, the uptake of antigen in vitro is demonstrable only at low temperatures and in the absence of serum. Finally,

cytophilic antibody from guinea pigs with delayed-type hypersensitivity has so far been shown to become attached only to macrophages and not to other cells, whereas the hypothesis above would require that cytophilic antibody be attached to serosal cells as well.

The rapid reversibility of cell clumping in vitro is rather surprising if the cells are in fact more sticky. One possible reason for this is that the cells adhere more readily or more firmly to glass than to each other. Alternatively, it may be that the maintenance of cell-to-cell adhesion requires some co-factor which was not present in the serum used in the culture medium.

A question which naturally arises is whether increased macrophage stickiness can explain other manifestations of delayed-type hypersensitivity. It may be, for example, that the accumulation and persistence of macrophages at the site of a delayed skin reaction or in the bed of a homograft being rejected depend on the firm attachment of these cells to the tissues at such sites.

Delayed-type hypersensitivity to tuberculin was induced in guinea pigs by vaccination with 250. The effects of several drugs on the response of peritoneal exudate cells to tuberculin (175) and on

C. REACTIONS TO ANTIGEN OF PERITONEAL CELLS OF GUINEA PIGS WITH DELAYED-TYPE HYPERSENSITIVITY

were followed by the virtually complete absence of response from the exudates (the macrophage reaction). In contrast to the partial loss of lymphocytes and a marked reduction in the number of polymorphonuclear leukocytes, the

PAPER V.

THE EFFECTS OF ANTICOAGULANTS AND OTHER DRUGS ON CELLULAR AND CUTANEOUS REACTIONS TO ANTIGEN IN GUINEA PIGS WITH DELAYED-TYPE HYPERSENSITIVITY

treated with cortisone acetate or heparin were almost completely inhibited in animals treated with procaine. The effect of peritoneal cellular responses were variably but only slightly affected by these drugs. Delayed-type reactions to PPD were partly inhibited in animals treated with heparin, sodium warfarin or cortisone acetate and were strongly inhibited in animals treated with a combination of sodium warfarin and cortisone acetate. Histological examination of the skin test sites of treated animals showed that the response to PPD was almost completely inhibited in animals treated with heparin, sodium warfarin or cortisone acetate.

SUMMARY

Delayed-type hypersensitivity to tuberculin was induced in guinea pigs by vaccination with BCG. The effects of several drugs on the responses of peritoneal exudate cells to tuberculin (PPD) and on delayed skin reactions to PPD were investigated. In untreated animals intraperitoneal injections of PPD were followed by the virtually complete loss of macrophages from the exudates (the macrophage disappearance reaction), the partial loss of lymphocytes and a marked increase in the number of polymorphs in the exudates. The macrophage disappearance reaction was markedly or completely inhibited in animals treated with the anticoagulant drugs heparin or sodium warfarin, very slightly inhibited in animals treated with cortisone acetate or Phenergan and not inhibited in animals treated with reserpine. The other peritoneal cellular responses were variably but only slightly affected by these drugs. Delayed skin reactions to PPD were partly inhibited in animals treated with heparin, sodium warfarin or cortisone acetate and more strongly inhibited in animals treated with a combination of sodium warfarin and cortisone acetate. Histological examination of the skin test sites of untreated animals and of animals treated with sodium warfarin and/or cortisone

acetate showed that the accumulation of macrophages was more markedly inhibited in animals treated with sodium warfarin than in animals treated with cortisone alone. No correlation could be established between the effect of treatment with sodium warfarin on the macrophage disappearance reaction, on blood coagulation and on serum complement levels.

INTRODUCTION

When antigen is injected intraperitoneally, intravenously or subcutaneously into guinea pigs with delayed-type hypersensitivity, the free floating macrophages of peritoneal exudates form clumps and then disappear almost completely. This macrophage disappearance reaction is characteristic of guinea pigs with delayed-type hypersensitivity, with or without Arthus hypersensitivity, and does not occur in normal guinea pigs or guinea pigs with pure Arthus hypersensitivity (Nelson and Boyden, 1963 a). Evidence presented elsewhere suggests that the loss of macrophages is due to their adherence, singly and in clumps, to the lining of the peritoneal cavity (Nelson and North, 1963). It was suggested that when antigen is injected into animals with delayed-type hypersensitivity the macrophages become more sticky and that this increased stickiness causes their adherence to each other and to the peritoneal endothelium. It is not known how this reaction is brought about, but one hypothesis which was considered was that it was due to a substance released when the cells of hypersensitive animals reacted with antigen. This possibility was investigated in the following way. Peritoneal fluid was obtained from hypersensitive animals either injected or

not injected with antigen. The fluid was allowed to clot in vitro and the "serum" obtained was injected into normal guinea pigs. It was found that the macrophages disappeared from the exudates of recipient animals whether the "serum" came from hypersensitive donors injected with antigen or from control animals. On the other hand, if the peritoneal fluid was prevented from clotting by the addition of heparin, injection of the "plasma" into normal animals was not followed by disappearance of the macrophages. These results suggested that there might be a relationship between clotting mechanisms and the response of macrophages to antigen. To test this possibility the effects were examined of anticoagulant drugs (heparin and sodium warfarin) on the response to tuberculin in vivo of the peritoneal macrophages of guinea pigs with delayed-type hypersensitivity to tuberculin. The effects were also examined of some other drugs known to inhibit some manifestations of different forms of hypersensitivity. These included cortisone, the antihistamine agent Phenergan and the anti-serotonin agent reserpine. The results of these experiments were briefly described elsewhere (Nelson, 1963) and indicated that the macrophage disappearance reaction could be inhibited by anticoagulants. A detailed description of these experiments is presented here

together with a description of the effects of anticoagulants and cortisone on delayed skin reactions to tuberculin and some experiments on the mode of action of sodium warfarin.

They were fed on standard guinea pig pellets plus hay and with greens. Ascorbic acid was added to the drinking water. Albino female guinea pigs of 100-150 g. were used for skin testing.

Sensitization. The guinea pigs were sensitized intraperitoneally with 0.1 mg. PPD (Commercial Drug Laboratories, N. York) 4 to 6 weeks before use.

Exudates. All experiments on the peritoneal exudate response to antigen were carried out on guinea pigs in which peritoneal exudates had been induced by glycerol. Four days before use the animals were injected intraperitoneally with 10 ml. of a solution containing 0.5% w/v ester glycogen (British Drug House) in 0.9% sterile normal saline.

Effect of antigen injections on peritoneal exudates. The purified protein derivative of tuberculin (PPD) was obtained from the Statens Serum Institut, Copenhagen. Batch RT 25 was used in some experiments and Batch RT 22 in most; these batches were of similar potency. Ten micrograms PPD in 1 ml. saline solution were injected

MATERIALS AND METHODS

Animals. Albino male guinea pigs weighing 580 ± 6 gm. were used for all experiments on peritoneal exudates. They were fed on standard guinea pig pellets supplemented with greens. Ascorbic acid was added to their drinking water. Albino female guinea pigs of the same size were used for skin testing.

Sensitization. The guinea pigs were injected intradermally with 0.1 mg. BCG (Commonwealth Serum Laboratories, Melbourne) 4 to 6 weeks before use.

Exudates. All experiments on the peritoneal cellular response to antigen were carried out on guinea pigs in which peritoneal exudates had been induced by glycogen. Four days before use the animals were injected intraperitoneally with 10 ml. of a solution containing 0.04 mg. oyster glycogen (British Drug Houses) per ml. sterile normal saline.

Effect of antigen injections on peritoneal exudates. The purified protein derivative of tuberculin (PPD) was obtained from the State Serum Institute, Copenhagen. Batch RT 23 was used in some experiments and Batch RT 22 in most; these Batches were of similar potency. Ten micrograms PPD in 1 ml. Hanks' solution were injected

intraperitoneally into hypersensitive guinea pigs. The exudates were harvested, usually $2\frac{1}{2}$ hours later, and total and differential cell counts were performed as described previously (Nelson and Boyden, 1963 a) except that the smears were stained with MacNeal's tetrachrome stain. The cells were classified as macrophages, lymphocytes or polymorphs. Eosinophils were classified separately from other polymorphs but only total polymorph counts are presented in the Tables below. Basophils or mast cells were very rare. The total numbers of each cell type (absolute cell counts) in the exudates were calculated from the total and differential cell counts.

Animals which had received intraperitoneal injections of PPD are referred to below as "injected animals"; those which had not received PPD are referred to as "uninjected".

Drugs. Heparin ("Pularin", Evans) was obtained and injected as a solution containing 1,000 International Units per ml.

Warfarin sodium (3-(acetonylbenzyl)-4-hydroxycoumarin sodium) was the gift of Evans Medical Ltd. It was supplied as a powder which was stored in the dark at $0-5^{\circ}\text{C}$. It was dissolved in saline to the required concentration just before use.

Cortisone acetate (Andrews Laboratories, Sydney)

was obtained and injected as an aqueous suspension at a concentration of 25 mg./ml.

Reserpine ("Serpasil", Ciba) was obtained and injected as a solution at a concentration of 1 mg./ml.

Phenergan (mepyramine maleate, May and Baker) was obtained as a solution which was diluted in sterile normal saline and injected at a concentration of 2.5 mg./ml.

The drugs were injected intravenously (i.v.), intraperitoneally (i.p.) or intramuscularly (i.m.) as indicated below. Intravenous injections were made into the ear veins of guinea pigs under light ether anaesthesia. Intramuscular injections were made into the thighs.

Animals which had received injections of drugs are referred to below as "treated animals"; those which had not received drugs are referred to as "untreated".

Estimation of blood coagulation defects. The Thrombotest method (Owren, 1959) was used, the reagent being obtained from Evans Medical Ltd. This method measures the amounts of Factor II (prothrombin), Factor VII (proconvertin), Factor IX (Christmas factor) and Factor X (Stuart-Prower factor). Blood was obtained from an incision in the ear and 0.05 ml. was added to 0.25 ml. of the Thrombotest reagent at 37°C. The time between the addition of blood to the reagent and coagulation of the mixture was measured

with a stop watch.

Estimation of serum complement (C') activity. The assay used was based on the agglutination of human erythrocytes by antigen-antibody-complement complexes reacting in immune adherence (Nishioka, 1963; Nelson, 1963 b). All dilutions were made in veronal buffered saline, pH 7.4-7.6, containing $0.00015 \text{ M Ca}^{++}$, and 0.0005 M Mg^{++} and 0.5% gelatin (GVB⁺⁺) (Nishioka, 1963). The antigen was twice crystallized hen ovalbumin (Sigma Chemical Company). Anti-ovalbumin antibody was prepared in rabbits immunized according to a standard schedule (Boyden, 1962). The antiserum was heated to 56°C for 30 minutes before use. For the C' estimations, blood was taken from guinea pigs by cardiac puncture and allowed to clot at room temperature. The clots were allowed to retract at $0-5^{\circ}\text{C}$ for 2 hours and the sera were separated by centrifugation at 0°C . The sera were stored in sealed glass ampoules at -20°C and thawed just before use, when they were absorbed twice at $0-5^{\circ}\text{C}$ with equal volumes of washed packed human erythrocytes. Serial twofold dilutions of the absorbed sera were made in GVB⁺⁺. Venous blood (Group O, Rh negative) was obtained from a human donor and stored in Alsever's solution at $0-5^{\circ}\text{C}$. An aliquot was centrifuged and washed twice in saline, care being taken to remove the buffy coat. The erythrocytes were resuspended in GVB⁺⁺ to a concentration of

1%. The optimal concentrations of antigen and antiserum were determined in preliminary assays with normal guinea pig C'. In the assay of C' in the sera of experimental animals, 0.2 ml. of ovalbumin solution (0.001 mg./ml.) and 0.2 ml. antiserum diluted 1:400 were mixed in small serological tubes and incubated briefly at 37°C. Dilutions of the guinea pig serum to be tested were added and the mixtures were incubated at 37°C for 10 min.; 0.2 ml. of the erythrocyte suspension was then added to each tube and the mixtures were incubated at 37°C for 30 min., with intermittent shaking for the first 10 min. They were then allowed to stand at room temperature until the erythrocytes had settled. The haemagglutination patterns were read and graded as + to +++. The endpoint was taken as the last tube showing ++ or greater hemagglutination. The results were expressed in arbitrary units of IA_{50} per ml.; for example, if ++ haemagglutination occurred in the tube containing 0.2 ml. guinea pig serum diluted 1:160, the C' titre was 800 IA_{50} per ml. In all these assays control mixtures were set up from which 1, 2 or all 3 of the reagents, antigen, antibody and C' were omitted; no haemagglutination was seen in these controls.

Skin tests. Albino female guinea pigs were used. The tests were performed and read as described previously

(Nelson and Boyden, 1963 a), 10 micrograms PPD being injected intradermally in 0.1 ml. saline. The results are expressed as specific increase in skin thickness, this being the mean increase in thickness of a double fold of skin at the test site in hypersensitive animals, less the mean increase in skin thickness of similarly injected normal animals.

Histological examination of skin test sites. After the last reading of the skin tests at 48 hours, pieces of skin (full thickness) including the centres of the reactions were excised, fixed in formal saline and processed by routine methods. Sections were cut 5 microns thick and stained with haematoxylin and eosin.

Statistical evaluation. The significance of differences in the peritoneal cell counts between groups of animals was evaluated by Student's t test. Differences were considered to be significant when $p \leq 0.01$.

RESULTS

The Effect of Heparin on the Peritoneal Cellular Response to Antigen

These experiments were designed to determine whether treatment with heparin could inhibit or reverse the macrophage disappearance reaction when PPD was injected into hypersensitive guinea pigs.

Groups of BCG-vaccinated guinea pigs with peritoneal exudates were injected with different doses of heparin i.p. either before or after being injected i.p. with 10 micrograms PPD. Others received heparin i.v. before the PPD. Appropriate controls were included, as shown in Table 1. This Table shows the cell contents of the peritoneal exudates of the heparin-treated and control animals.

The intraperitoneal injection of PPD alone was followed by a very marked fall in the macrophage contents of the exudates; this was apparent in exudates harvested 1 or $2\frac{1}{2}$ hours after injection. A significant fall in the lymphocyte counts and a significant rise in the polymorph counts were apparent in the exudates harvested $2\frac{1}{2}$ hours after the injection of PPD.

In all the heparin-treated animals the macrophage contents of the exudates $2\frac{1}{2}$ hours after the injection of

TABLE 1.

Effect of Heparin on Peritoneal Cellular Reactions to PPD

Heparin Dose, route of injection and time of injection	PPD dose injected, time before harvest	Number of Animals	Absolute Cell Count x 10 ⁻⁴ Mean ± Standard Error		
			Macrophages	Lymphocytes	Polymorphs
None	None	13	2608 [±] 223	1137 [±] 193	155 [±] 33
None	10 micro- grams, 1 hr	6	237 [±] 85	1092 [±] 316	254 [±] 91
None	10 micro- grams, 2½ hrs	20	162 [±] 20	596 [±] 96	3508 [±] 257
100 units, i.p., 2½ hrs before harvest	None	6	3209 [±] 366	1104 [±] 346	1231 [±] 92
100 units, i.p., 10 min before PPD	10 micro- grams, 2½ hrs	6	683 [±] 164	371 [±] 71	4076 [±] 966
100 units, i.p., 1 hr after PPD	10 micro- grams, 2½ hrs	6	526 [±] 255	403 [±] 77	4614 [±] 517
250 units, i.p., 2½ hrs before harvest	None	6	2846 [±] 310	968 [±] 222	606 [±] 224
250 units, i.p., 10 min before PPD	10 micro- grams, 2½ hrs	6	1184 [±] 85	619 [±] 107	3956 [±] 511
250 units, i.p., 1 hr after PPD	10 micro- grams, 2½ hrs	6	814 [±] 347	674 [±] 246	4249 [±] 656
500 units, i.p., 2½ hrs before harvest	None	6	2083 [±] 246	564 [±] 53	1564 [±] 392
500 units, i.p., 10 min. before PPD	10 micro- grams, 2½ hrs	8	1086 [±] 176	413 [±] 102	3091 [±] 329
500 units, i.p., 1 hr after PPD	10 micro- grams, 2½ hrs	6	481 [±] 112	676 [±] 173	5716 [±] 499
1000 units, i.p., 2½ hrs before harvest	None	10	1759 [±] 233	448 [±] 55	1034 [±] 343
1000 units, i.p., 10 min before PPD	10 micro- grams, 2½ hrs	8	2228 [±] 412	1007 [±] 242	2575 [±] 436
1000 units, i.p., 1 hr after PPD	10 micro- grams, 2½ hrs	6	510 [±] 84	744 [±] 229	4373 [±] 545
None; saline i.v., 3½ hrs before harvest	10 micro- grams, 2½ hrs	6	137 [±] 16	655 [±] 139	3931 [±] 683
2000 units, i.v., 3½ hrs before harvest	None	7	3243 [±] 286	1076 [±] 134	332 [±] 162
2000 units, i.v., 1 hr before PPD	10 micro- grams, 2½ hrs	6	1923 [±] 316	380 [±] 52	3810 [±] 446

PPD were significantly higher than those in untreated animals injected with PPD. In the animals injected i.p. with heparin 10 minutes before the PPD the highest counts occurred in those receiving the highest doses. High doses of heparin alone caused a slight but significant fall in the macrophage counts. In animals injected i.p. with 1,000 units of heparin there was no significant difference between the macrophage counts in the exudates of those receiving heparin alone and those receiving PPD 10 minutes after the heparin. In animals injected i.p. with smaller doses of heparin the injection of PPD 10 minutes later was followed by significant falls in the macrophage counts but these were less marked than in untreated controls. Similarly in animals receiving 2,000 units of heparin i.v. the injection of PPD 1 hour later was followed by a significant fall in the macrophage contents of the exudates but this was not marked: the macrophage counts were more than 10 times as high as in control animals receiving no treatment or saline i.v. 1 hour before the PPD injections. In animals injected with heparin i.p. 1 hour after the PPD the macrophage counts were 3 to 5 times as high as in animals receiving PPD alone $2\frac{1}{2}$ hours before the exudates were harvested; these differences were statistically significant. The macrophage contents of the exudates of these animals were also significantly higher

than those of animals injected with PPD alone 1 hour before the exudates were harvested, i.e., at the same time as heparin was injected into animals of other groups. These differences were not related to the dose of heparin injected.

These results indicate that heparin injected before antigen can strongly inhibit the macrophage disappearance reaction and that heparin injected after the antigen can partially reverse the reaction.

The fall in the lymphocyte counts in the exudates of BCG-vaccinated guinea pigs after the injection of PPD was less clearly affected by heparin. In animals injected i.p. with heparin alone in doses of 500 or 1,000 units the lymphocyte counts were significantly lower than in untreated animals. In animals receiving these doses of heparin followed by PPD the lymphocyte counts were not significantly different from those receiving heparin alone. In animals injected i.p. with smaller doses of heparin or i.v. with 2,000 units of heparin, either alone or followed by PPD, the lymphocyte counts were not significantly different from those of the untreated controls. In animals injected with heparin after the PPD injections the lymphocyte counts were not significantly different from those injected with PPD alone. It is concluded from these results that heparin injected i.p. in large doses induces a slight loss

of lymphocytes from the exudates but also prevents any further loss after the injection of antigen.

The differences in polymorph counts between heparin-treated and untreated animals injected with PPD were small and their meaning is not clear. The polymorph counts in the exudates of BCG-vaccinated animals receiving 1,000 units of heparin i.p. before PPD injections were significantly lower than in those of untreated animals injected with PPD. In animals receiving 2,000 units of heparin i.v. or smaller doses i.p. before the PPD injections the polymorph counts did not differ significantly from those of untreated animals injected with PPD. In animals receiving heparin alone i.p. the polymorph counts were significantly higher than in untreated animals; these differences were not related to the dose of heparin. In the animals injected i.p. with 100 or 500 units of heparin 1 hour after the PPD the polymorph counts were significantly higher than in the untreated animals injected with PPD.

The effect of Sodium Warfarin on the Peritoneal Cellular Response to Antigen

It was clear from the results of the experiments just described that adequate doses of heparin could prevent the disappearance of macrophages from the peritoneal

exudates of BCG-vaccinated guinea pigs injected with PPD. The effect of another anticoagulant was examined; sodium warfarin was chosen because it is soluble in water and therefore suitable for intravenous injection and because its mode of action is different from that of heparin. Like other coumarin derivatives which are Vitamin K analogues, it inhibits the synthesis of prothrombin (Factor II), proconvertin (Factor VII), Christmas factor (Factor IX) and Stuart-Prower factor (Factor X) (Owren, 1959; Noren and Quick, 1963). The effect of sodium warfarin on blood coagulation is delayed since some time is required for the blood levels of these factors to fall after their synthesis has been inhibited.

BCG-vaccinated guinea pigs with peritoneal exudates were injected i.v. with sodium warfarin according to either of two schedules. One group of 14 guinea pigs received 20 mg. 2 days before and 10 mg. 1 day before the exudates were harvested; 8 of these animals were injected i.p. with 10 micrograms PPD $2\frac{1}{2}$ hours before the exudates were harvested. A second group of 16 guinea pigs received 10 mg. 3 days before and 5 mg. 2 days and 1 day before the exudates were harvested; 9 of these animals were injected i.p. with 10 micrograms PPD $2\frac{1}{2}$ hours before the exudates were harvested. Control animals received 3 daily i.v. injections of sterile saline. The cell contents of the

TABLE 2.

Effect of Warfarin on Peritoneal Cellular Reaction
to PPD

Intravenous injections	PPD i.p. 2½ hrs before harvest	No. of animals	Absolute Cell Count Mean ± Standard Error		
			Macrophages	Lymphocytes	Polymorphs
None	None	13	2608 [±] 223	1137 [±] 193	155 [±] 33
None	10 micro- grams	20	162 [±] 20	596 [±] 96	3508 [±] 257
Saline ^a	10 micro- grams	6	245 [±] 53	744 [±] 119	5301 [±] 782
Warfarin ^b	None	6	3157 [±] 550	1201 [±] 153	46 [±] 16
Warfarin ^b	10 micro- grams	8	2145 [±] 327	574 [±] 135	4246 [±] 317
Warfarin ^c	None	7	2982 [±] 250	1084 [±] 347	71 [±] 31
Warfarin ^c	10 micro- grams	9	2072 [±] 161	739 [±] 339	3467 [±] 417

^a 1 ml. saline i.v. 3 days, 2 days and 1 day before harvest.

^b Warfarin i.v. 20 mg. 2 days before and 10 mg. 1 day before harvest.

^c Warfarin i.v. 10 mg. 3 days before, 5 mg. 2 days and 1 day before harvest.

peritoneal exudates of these animals and of untreated animals injected or not injected with PPD are shown in Table 2.

The macrophage contents of the exudates of both groups of warfarin treated guinea pigs injected with PPD were slightly but significantly lower than those of treated guinea pigs not injected with PPD. They were, however, 8 to 12 times higher than those of BCG-vaccinated animals injected with PPD alone or with saline and PPD, these differences being statistically significant. There was no difference in the macrophage contents of the exudates between treated and untreated animals not injected with PPD. It is apparent that pretreatment of hypersensitive guinea pigs with sodium warfarin resulted in a marked though not complete inhibition of the macrophage disappearance reaction to injected antigen.

The lymphocyte counts in treated animals either injected or not injected with PPD were not significantly different from those in the respective control animals.

As in the case of heparin, there were differences in the polymorph counts between warfarin-treated and untreated animals but the differences were slight and their meaning is not clear. The polymorph contents of the exudates of warfarin-treated animals not injected with PPD were slightly but significantly lower than those of

untreated, uninjected animals. The polymorph counts in animals injected with PPD after i.v. injections of either saline or warfarin (20 mg. and 10 mg.) were significantly higher than in untreated animals injected with PPD; those of animals injected with PPD after treatment with warfarin (10 mg., 5 mg. and 5 mg.) were not significantly different from those of injected untreated animals.

Other Effects of Sodium Warfarin

It was thought that some clue to the way in which anticoagulants act in inhibiting the macrophage disappearance reaction might be obtained by comparing the effect of sodium warfarin on this reaction with its effect on blood coagulation and serum complement levels. It is not known whether or not C' is involved in any of the manifestations of delayed-type hypersensitivity but an association between blood coagulability and serum C' activity has been known for some time. Heparin, hirudin and bile have both anticoagulant and anticomplementary effects (Pfannenstiel, 1927; Busing and Zuzak, 1943). Vitamin K deficiency is associated with decreased levels of both prothrombin and C' (Busing and Zuzak, 1943; Weber, Wiss and Isliker, 1963). It was conceivable that the effect of sodium warfarin treatment on the macrophage disappearance reaction might be due to lowered C' activity.

Serum for C' estimations was obtained from 6

TABLE 3.

Comparison of the Effects of Warfarin on the Peritoneal Macrophage Response,
Blood Coagulation and Serum Complement

Treatment	Guinea Pig number	Macrophage Content of Exudates		Thrombotest, time, seconds	Serum C', IA ₅₀ /ml.	
		Absolute Count x 10 ⁻⁴	% decrease ^a after PPD		Before PPD	After PPD
None, no PPD	Mean of 13	2608 [±] 223	-	-	-	-
None, PPD 10 micro-grams	3152	146	94	56	800	800
	3154	554	79	52	800	400
	3161	345	87	60	400	800
	3163	179	93	63	800	800
	3169	221	92	50	800	800
	3176	988	62	55	400	400
Warfarin ^b , no PPD	Mean of 6	3157 [±] 550	-	-	-	-
Warfarin ^b , PPD 10 micro-grams	3171	2065	34	200	-	200
	3173	1332	58	165	-	800
	3174	1959	38	140	-	1600
	3182	4047	0	90	-	800
Warfarin ^c , no PPD	Mean of 7	2982 [±] 250	-	-	-	-
Warfarin ^c , PPD 10 micro-grams	3166	2640	15	230	-	400
	3178	2368	21	290	-	400
	3184	1750	41	300	-	100

^a Compared with mean count of peritoneal macrophages in animals not injected with PPD.

^b Warfarin i.v. 20 mg. 2 days before and 10 mg. 1 day before harvest.

^c Warfarin i.v. 10 mg. 3 days before, 5 mg. 2 days and 1 day before harvest.

untreated BCG-vaccinated guinea pigs just before they were injected i.p. with 10 micrograms PPD and $2\frac{1}{2}$ hours later just before the exudates were harvested. Serum was also obtained just before the exudates were harvested from 7 guinea pigs treated with sodium warfarin and injected i.p. with 10 micrograms PPD $2\frac{1}{2}$ hours earlier; blood was not taken from these animals before the PPD injections because of their pronounced bleeding tendency. C' levels in the sera were later estimated by the immune adherence technique described above. Blood was also taken from all the animals just before PPD was injected and the clotting time was measured with the Thrombotest reagent. The cell contents of the exudates harvested $2\frac{1}{2}$ hours after the injection of PPD were also measured. Table 3 shows the results of these measurements; the extent of the macrophage loss after the injection of PPD is expressed as the percent decrease in the macrophage counts compared with the counts of uninjected controls.

It can be seen that the Thrombotest time was considerably prolonged in all the warfarin treated animals but that there was no correlation between this prolongation and the degree of inhibition of the macrophage disappearance reaction.

There was no correlation between C' levels in the serum and the extent of the macrophage disappearance after

the injection of PPD. C' levels were low in only two of the warfarin treated animals and in neither of these was the macrophage disappearance reaction maximally inhibited. C' levels were the same before and after PPD injections in 4 of the 6 untreated animals; of the other 2, one showed a twofold rise and the other a twofold fall in titre.

The Effect of Cortisone Acetate on the Peritoneal Cellular Response to Antigen

Adrenocortical hormones have been frequently reported to inhibit delayed skin reactions to antigen in hypersensitive animals (e.g. Harris and Harris, 1950; Long and Miles, 1954). The effect of treatment with cortisone acetate on the macrophage disappearance reaction was therefore also investigated.

BCG-vaccinated guinea pigs received i.m. injections of cortisone acetate, 25 mg. daily, for 6 days. On the third day glycogen was injected intraperitoneally with glycogen. On the seventh day the exudates were harvested. Of the 13 animals treated with cortisone acetate, 7 were injected with 10 micrograms PPD i.p. $2\frac{1}{2}$ hours before the exudates were harvested. The cell contents of the exudates of these animals and of the exudates of untreated control animals injected or not injected with PPD are shown in Table 4.

TABLE 4.

Effect of Cortisone on Peritoneal Cellular Reactions to PPD

Intramuscular injections	PPD i.p. 2½ hrs before harvest	No. of animals	Absolute Cell Count Mean ± Standard Error			
			Macrophages	Lymphocytes	Total Polymorphs	Eosinophils
None	None	13	2608 [±] 223	1137 [±] 193	155 [±] 33	121 [±] 51
None	10 micro-grams	20	162 [±] 20	596 [±] 96	3508 [±] 257	149 [±] 50
Cortisone ^a	None	6	1085 [±] 231	319 [±] 66	16 [±] 5	3 [±] 4
Cortisone ^a	10 micro-grams	7	186 [±] 39	229 [±] 48	3468 [±] 450	15 [±] 9

^a

Cortisone acetate 25 mg. i.m. daily for 6 days before harvest.

There was no significant difference in the macrophage counts between cortisone-treated animals injected with PPD and untreated animals injected with PPD. The macrophage counts in cortisone-treated animals not injected with PPD were, however, significantly lower (by about 60%) than those of untreated animals not injected with PPD, so that the proportion of macrophages lost from the exudates after PPD injections was lower in the treated than in the untreated animals.

The lymphocyte content of the exudates was lower in the cortisone-treated than in the untreated animals and no significant further fall occurred after the injection of PPD.

The polymorph counts after the injection of PPD were the same in cortisone-treated as in untreated animals. The polymorph counts in the treated animals not injected with PPD, were, however, much lower than those in the untreated animals not injected with PPD. It can be seen that this is due largely to the absence of eosinophils. It is interesting to note that even in untreated animals eosinophils make no contribution to the polymorph response to PPD.

The occurrence of lymphopenia and eosinopenia indicate that the dose of cortisone acetate used was sufficient to produce pharmacological effects in the

guinea pigs.

The Effects of Phenergan and Reserpine on the Peritoneal Cellular Response to PPD

The antihistamine drug Phenergan (mepyramine maleate) has been reported to inhibit delayed skin reactions to tuberculin in hypersensitive guinea pigs (Pepys, 1955). The anti-serotonin agent reserpine has been found to inhibit some of the effects of bacterial endotoxins (Des Prez, Fallon and Hook, 1961); it has been noted that bacterial endotoxins can also cause the macrophage disappearance reaction (Nelson and Boyden, 1963 a). Although histamine and serotonin release are caused by complexes of antigen and humoral antibody (Humphrey and Jaques, 1955), it was considered worthwhile to investigate the effects of antagonists to these substances on the macrophage disappearance reaction.

BCG-vaccinated guinea pigs with peritoneal exudates were injected i.v. with 2.5 mg. Phenergan or 0.5 mg. reserpine $3\frac{1}{2}$ hours before the exudates were harvested. Of each group of 12 animals injected with either Phenergan or reserpine 6 were injected i.p. with 10 micrograms PPD $2\frac{1}{2}$ hours before the exudates were harvested. Control animals were injected i.v. with saline and i.p. with PPD at the same times. The cell contents of the peritoneal exudates of these animals and of untreated animals either

TABLE 5.

Effect of Phenergan and Reserpine on Peritoneal Cellular Reactions
to PPD

Intravenous injection	PPD 2½ hrs before harvest	No. of animals	Absolute Cell Count x 10 ⁻⁴ Mean ± Standard Error		
			Macrophages	Lymphocytes	Polymorphs
None	None	13	2608 [±] 223	1137 [±] 193	155 [±] 33
None	10 micro- grams	20	162 [±] 20	596 [±] 96	3508 [±] 257
Saline ^a	10 micro- grams	6	137 [±] 16	655 [±] 139	3931 [±] 683
Phenergan ^b	None	6	2700 [±] 253	1153 [±] 427	147 [±] 46
Phenergan ^b	10 micro- grams	6	367 [±] 160	618 [±] 128	4535 [±] 271
Reserpine ^c	None	6	2611 [±] 216	795 [±] 99	78 [±] 39
Reserpine ^c	10 micro-	6	148 [±] 39	898 [±] 455	4814 [±] 722

^a

Saline 1 ml. i.v. 3½ hr. before harvest

^bPhenergan 2.5 mg. i.v. 3½ hrs. before
harvest^c

Reserpine 0.5 mg. i.v. 3½ hrs. before harvest

injected or not injected with PPD are shown in Table 5.

The macrophage counts in the exudates of guinea pigs injected with Phenergan and PPD were significantly higher than those in untreated or in saline-treated animals injected with PPD. The differences were not nearly as great as those seen in the anticoagulant-treated animals. There were no significant differences in the counts of other cell types between the Phenergan-treated and the control animals.

In the case of reserpine the macrophage counts in the exudates of the treated animals, either injected or not injected with PPD, were not significantly different from those of the untreated controls. The lymphocyte counts in the reserpine-treated animals injected with PPD were very variable and did not differ significantly from those of reserpine-treated animals not injected with PPD or from those of untreated animals injected with PPD; reserpine treatment alone caused no fall in the lymphocyte counts. It is therefore not clear whether or not treatment with reserpine prevented the effect of PPD on the lymphocytes of hypersensitive guinea pigs. The polymorph counts in the exudates of reserpine-treated animals injected with PPD did not differ significantly from those of untreated animals injected with PPD, although those of reserpine-treated, uninjected animals were

slightly lower than those of untreated uninjected animals.

The Effects of Anticoagulants and Cortisone Acetate
on Skin Reactions to PPD

Although cortisone is known to inhibit skin reactions to antigen in animals with delayed-type hypersensitivity, the experiments described above showed that it did not inhibit the reaction to PPD of the peritoneal macrophages of hypersensitive guinea pigs to the same extent as did anticoagulants. It was therefore of some interest to compare the effects of cortisone acetate and anticoagulants on delayed skin reactions to PPD.

BCG-vaccinated female guinea pigs were injected as follows:

Group A - untreated controls;

Group B - Heparin 500 units i.p. $1\frac{1}{2}$ hours before skin testing and 200 units i.p. every 6 hours thereafter;

Group C - Heparin 1,500 units i.p. $1\frac{1}{2}$ hours before skin testing and 1,000 units i.p. every 6 hours thereafter;

Group D - Saline 1 ml. i.v. daily, beginning 48 hours before the skin test and continuing through the period of observation of the skin test;

Group E - Sodium warfarin 20 mg. i.v. 48 hours before the skin test and 10 mg. i.v. daily thereafter until the last skin test reading;

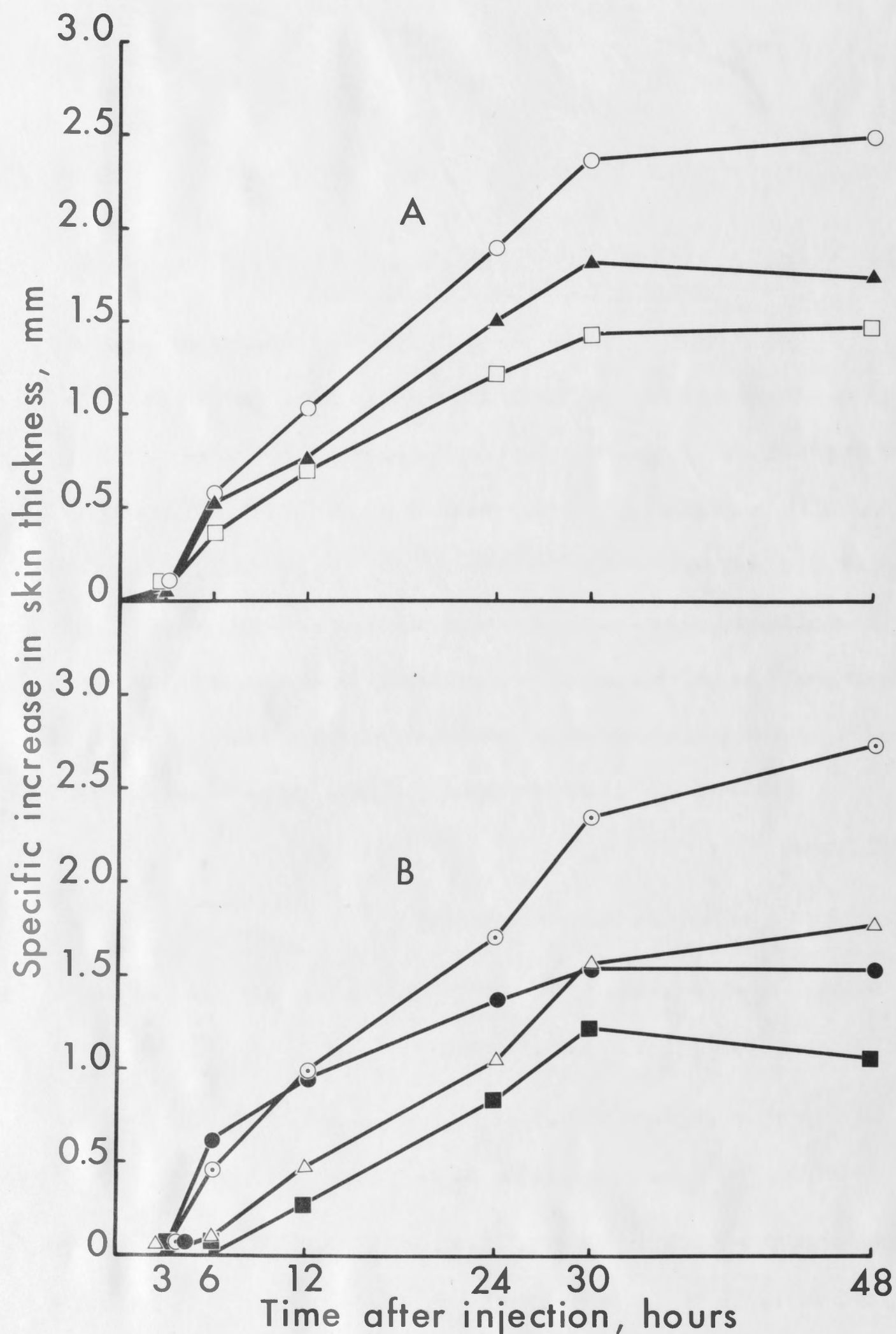


Figure 1. Skin reactions (mean specific increases in skin thickness) to PPD in BCG-vaccinated guinea pigs. A: ○—○, Group A (untreated); ▲—▲, Group B (heparin-treated); □—□, Group C (heparin-treated). B: ⊙—⊙, Group D (saline-injected); ●—●, Group E (warfarin-treated); △—△, Group F (cortisone-treated); ■—■, Group G (warfarin- and cortisone-treated).

Group F - Cortisone acetate 25 mg. i.m. 5 days before the skin test and daily thereafter until the last skin test reading;

Group G - Sodium warfarin as for Group E, plus cortisone acetate as for Group F.

The results of the skin tests, expressed as mean specific increases in skin thickness at the reaction sites, are shown graphically in Figure 1.

The control animals, either untreated or injected with saline, showed typical strong delayed reactions which were absent 3 hours after the injection of antigen, slight at 6 hours and increased progressively in size up to 30 to 48 hours. In the animals treated with heparin or sodium warfarin the delayed reactions were smaller than in the controls; inhibition was most marked at 48 hours. Treatment with cortisone produced slightly less inhibition of the reactions at 48 hours but more marked inhibition of the reactions at 6 to 12 hours. Combined treatment with cortisone and sodium warfarin produced even more marked inhibition of the reactions at all stages.

The skin test sites of control, warfarin- and cortisone-treated animals were biopsied 48 hours after the injection of antigen and examined histologically. In the control animals (Groups A and D) there was dense

infiltration with macrophages and polymorphs, the macrophage infiltration being particularly marked around blood vessels in the deep dermis and subdermal tissue (Figure 2). In the warfarin-treated animals (Group E) macrophage infiltration was much less marked though there was still much polymorph infiltration (Figure 3). In the cortisone-treated animals (Group F) the infiltration with both polymorphs and macrophages was reduced, but the characteristic perivascular accumulations of macrophages were still present (Figure 4). In the animals treated with both warfarin and cortisone (Group G) the cellular infiltration was grossly reduced and macrophages were very scanty (Figure 5).

It is clear that delayed skin reactions to PPD in hypersensitive guinea pigs were inhibited by treatment with anticoagulants to the same or a slightly greater extent than by treatment with cortisone acetate; they were most strongly inhibited by a combination of sodium warfarin and cortisone acetate. Furthermore, the accumulation of macrophages at the skin test sites was more markedly inhibited by sodium warfarin or by warfarin plus cortisone than by cortisone alone in the doses used.

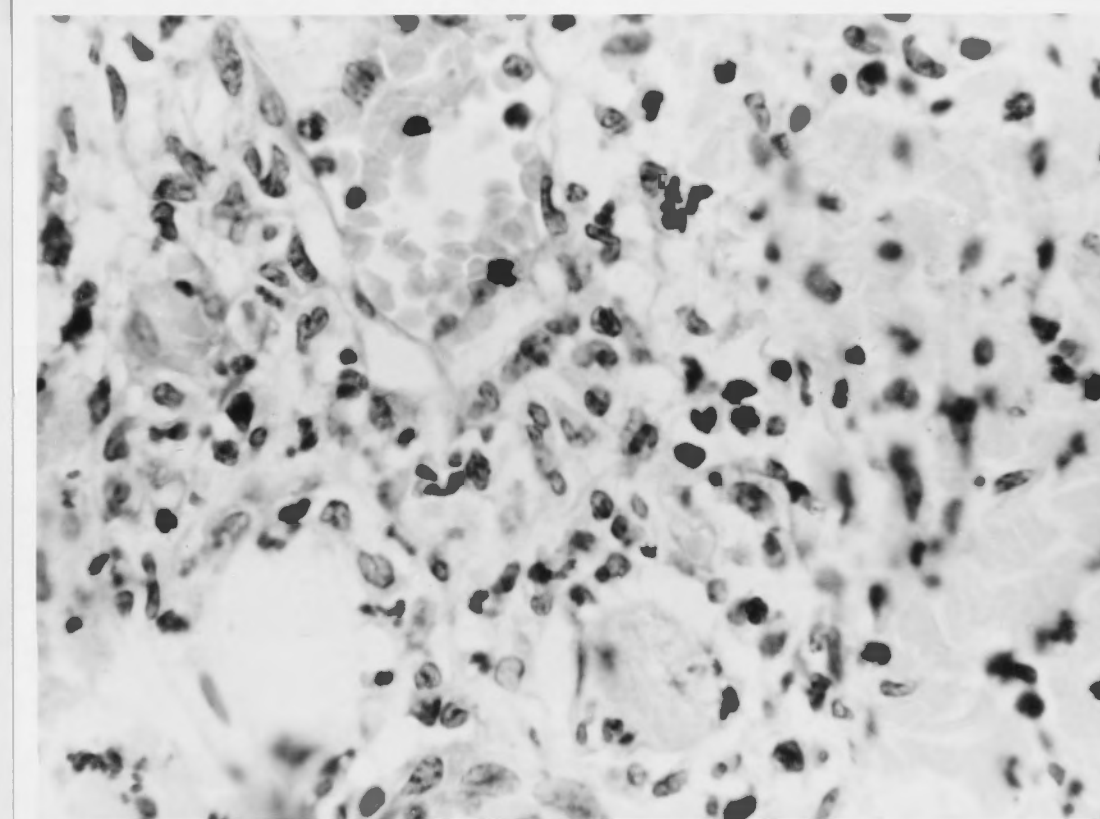
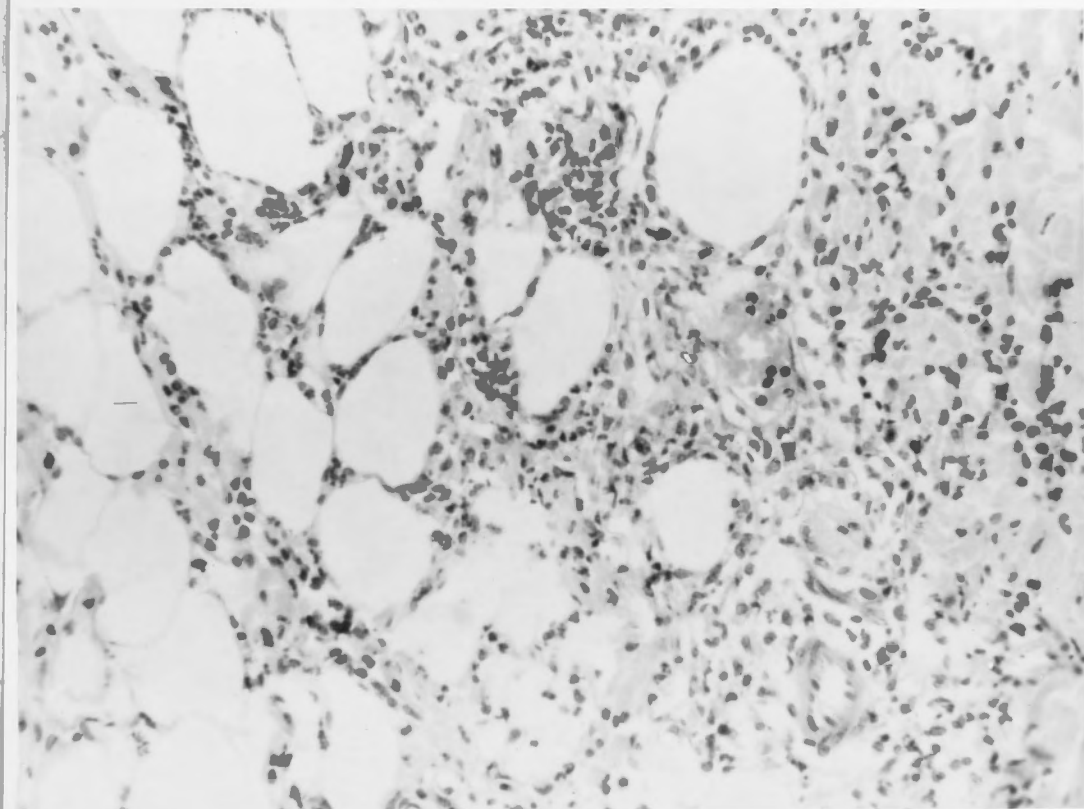


Figure 2. Tuberculin reaction in untreated guinea pig, 48 hrs.

There is marked infiltration of the deep dermis and subdermal tissue with mononuclear cells and some polymorphs. Mononuclear cells are most prominent in the perivascular areas (Haematoxylin and eosin; A, x 512; B, x 1280).

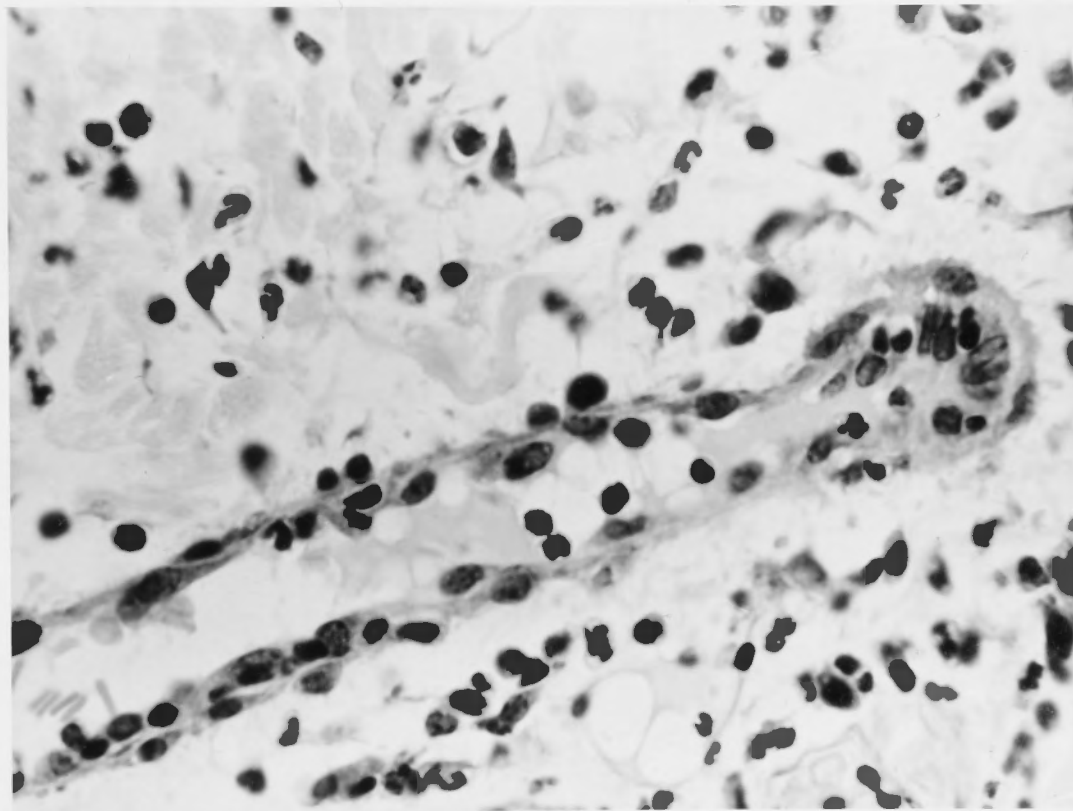
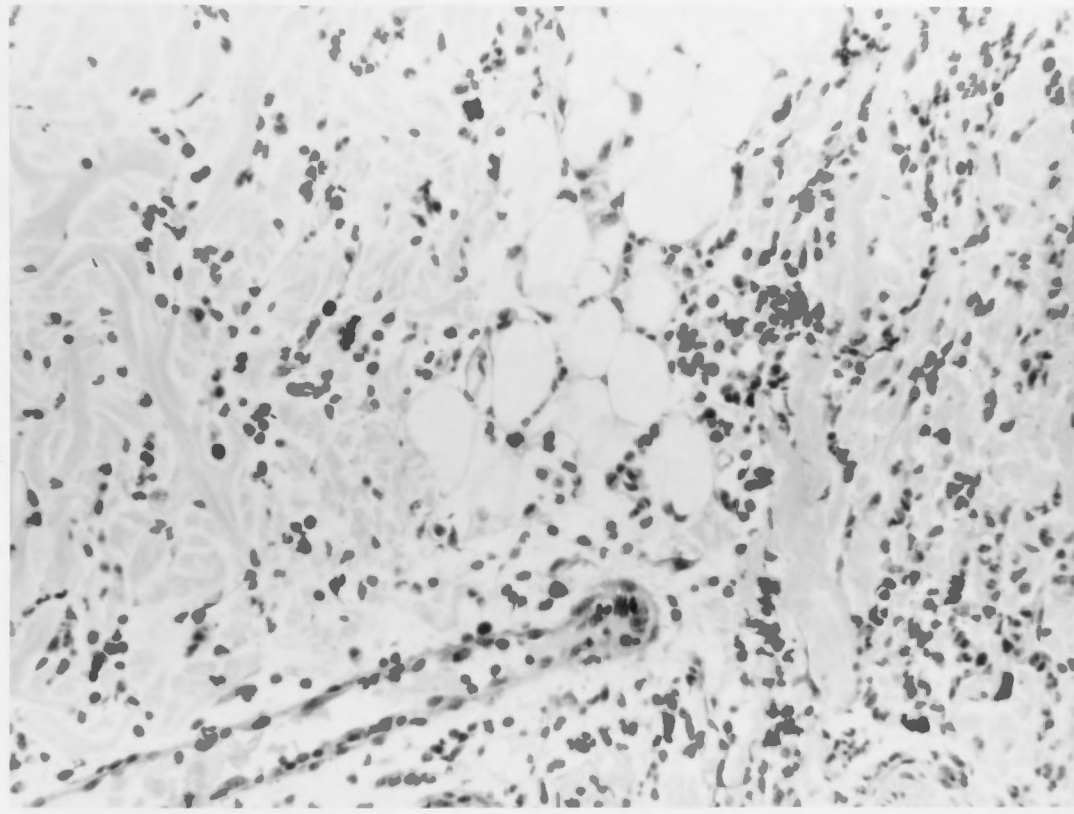


Figure 3. Tuberculin reaction in sodium warfarin-treated guinea pig, 48 hrs.

There is less infiltration of the deep dermis and subdermal tissue with mononuclear cells than in untrated guinea pigs. Perivascular infiltration is slight and the majority of the cells are polymorphs (Haematoxylin and eosin; A, x 512; B, x 1280).

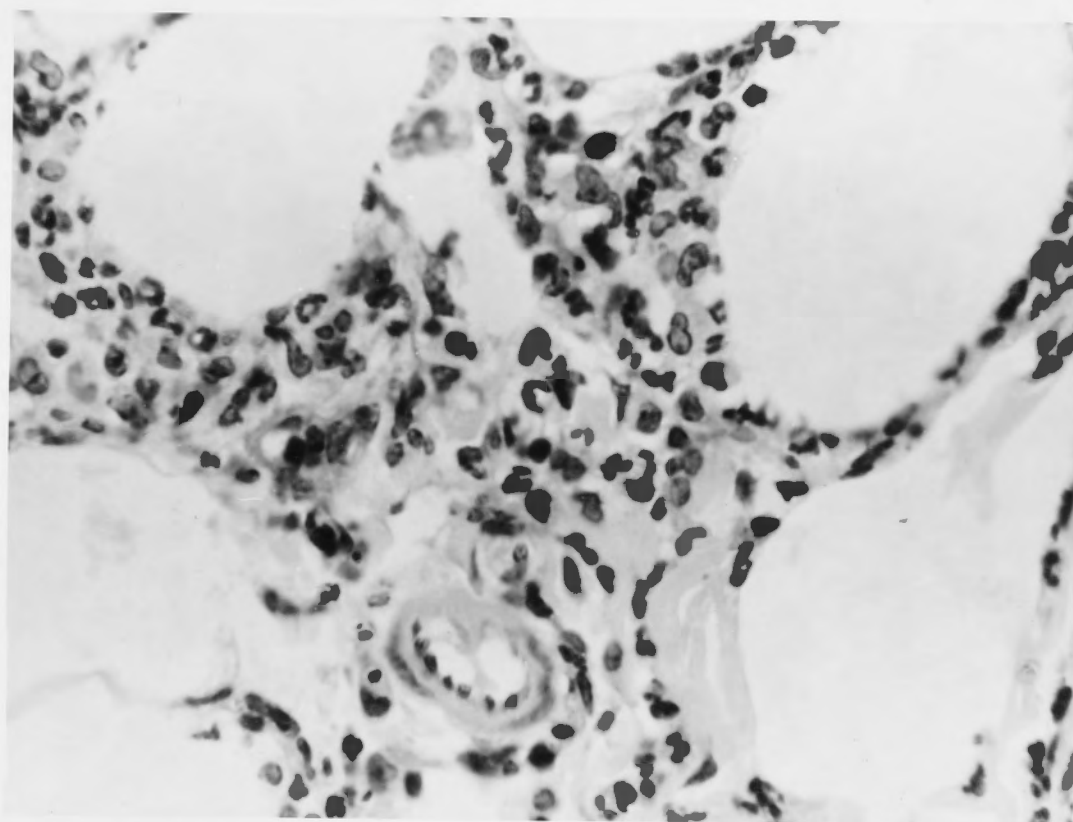
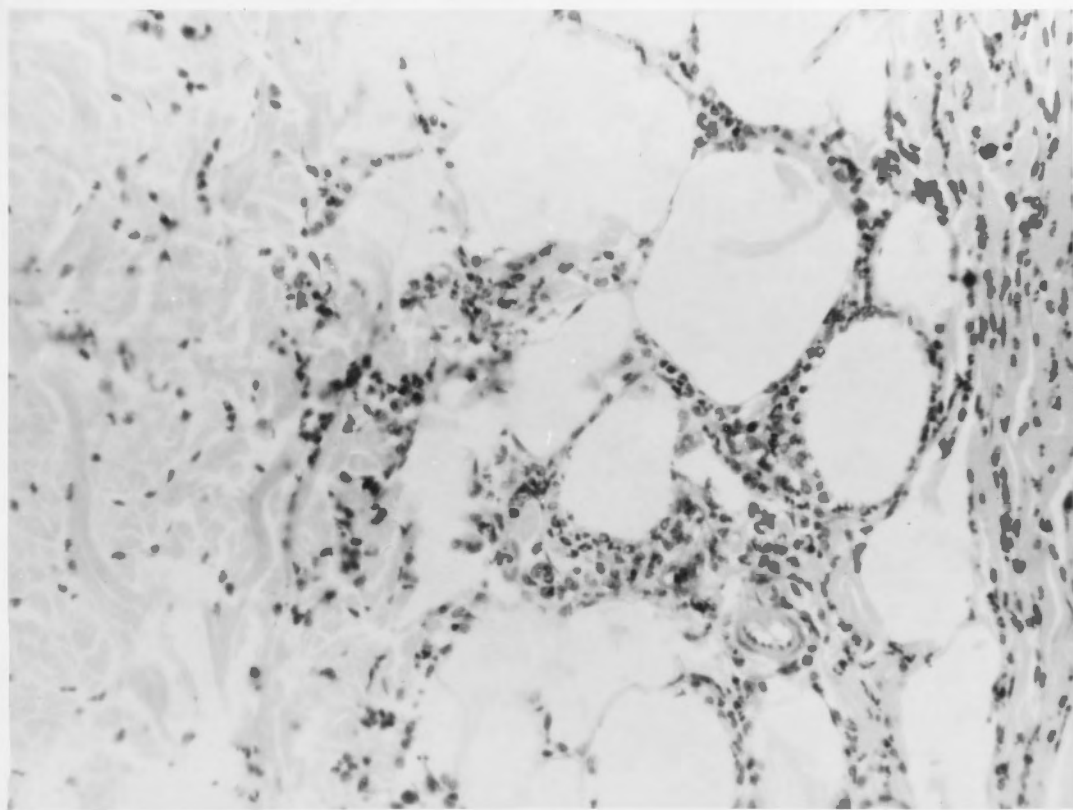


Figure 4. Tuberculin reaction in cortisone-treated guinea pig, 48 hrs.

The total cellular infiltration in the deep dermis and subdermal tissue is less marked than in untreated guinea pigs, but perivascular infiltration with mononuclear cells is still a feature (Haematoxylin and eosin; A, x 512; B, x 1280).

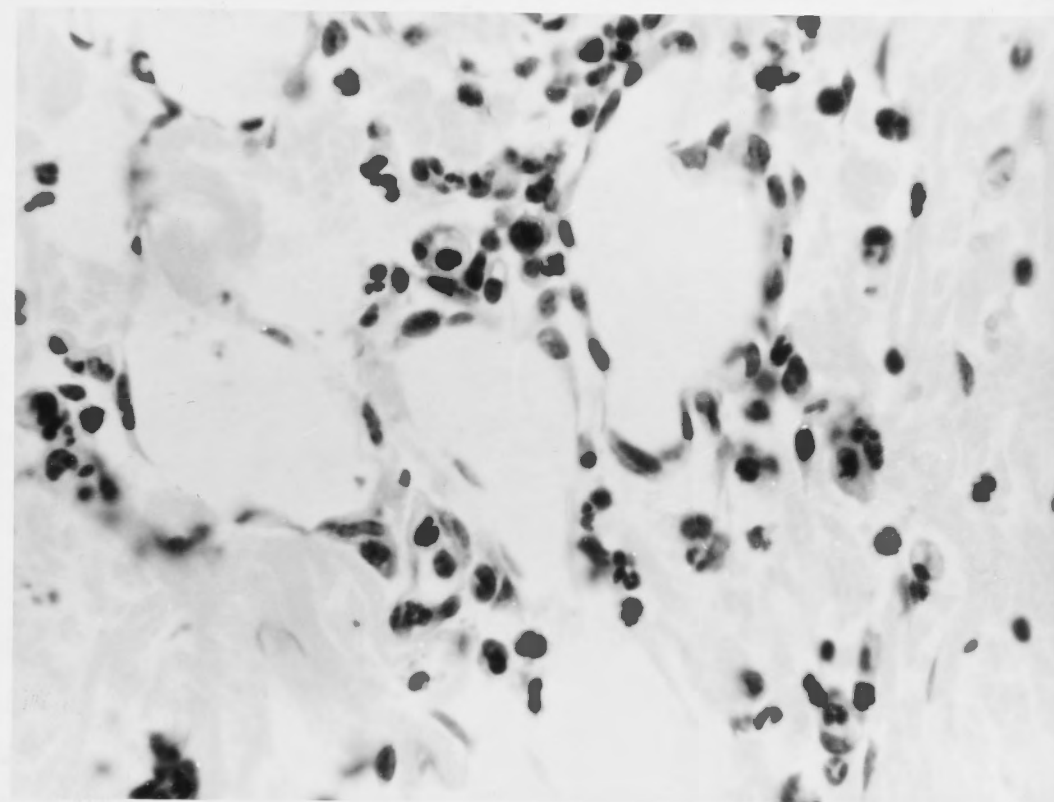
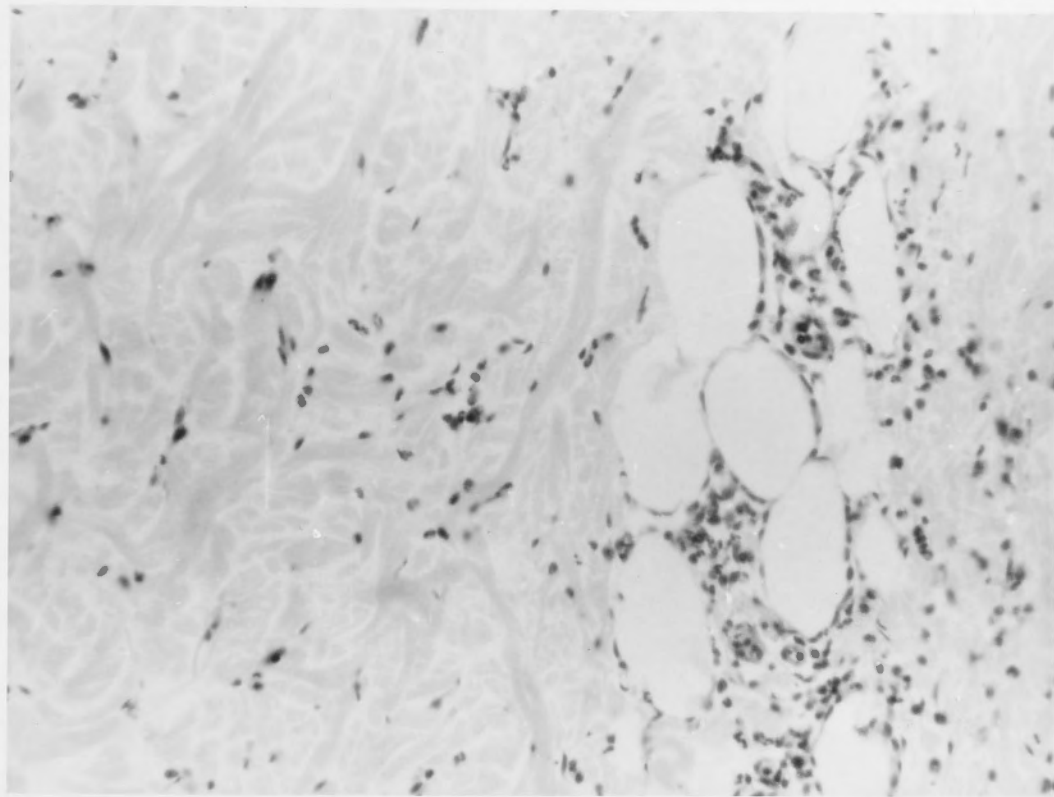


Figure 5. Tuberculin reaction in guinea pig treated with sodium warfarin and cortisone, 48 hrs. There is gross reduction in the number of cells infiltrating the deep dermis and subdermal tissue, compared with the reactions in untreated guinea pigs. Mononuclear cells are almost completely absent (Haematoxylin and eosin; A, x 512; B, x 1280).

TABLE 6.

Inhibition by Drugs of Peritoneal Macrophage Response
to PPD in BCG-Vaccinated Guinea Pigs

Drug and Dose	Percent Inhibition of Macrophage Response ^c
Heparin; 100 units i.p. 10 min. before PPD	16
Heparin; 250 units i.p. 10 min. before PPD	38
Heparin; 500 units i.p. 10 min. before PPD	49
Heparin; 1000 units i.p. 10 min. before PPD	100
Heparin; 2000 units i.v. 1 hr. before PPD	56
Warfarin; 20 mg, 10 mg i.v. ^a	66
Warfarin; 10 mg, 5 mg, 5 mg i.v. ^b	67
Cortisone; 25 mg/day i.m. for 6 days	12
Phenergan; 2.5 mg. i.v. 1 hr. before PPD	8
Reserpine; 0.5 mg. i.v. 1 hr. before PPD	0

^a Warfarin injected 2 days and 1 day before harvest

^b Warfarin injected 3 days, 2 days and 1 day before harvest

^c Calculated as:

$$\frac{\% \text{ fall in macrophage count in untreated animals} - \% \text{ fall in macrophage count in treated animals}}{\% \text{ fall in macrophage count in untreated animals}} \times 100$$

DISCUSSION

The effects on the macrophage disappearance reaction of the drugs tested in these experiments are summarized in Table 6. It is apparent that the most effective inhibitors were the anticoagulants heparin and sodium warfarin. The inhibition by cortisone was slight and was largely accounted for by the decrease in the number of macrophages initially present in the exudates. Phenergan, in the dose used, was even less effective and reserpine was ineffective. It was also found that heparin injected 1 hour after the antigen caused some reversal of the reaction, since the macrophage counts were higher than in animals injected with PPD alone either 1 hour or $2\frac{1}{2}$ hours before the exudates were harvested.

Minor differences in the responses to PPD of other cells in the peritoneal cavity were noted in treated animals. These were of less interest, as the most striking effect of the drugs was on the macrophage response. The lymphocyte counts were often very variable. The polymorph response is largely non-specific in that it has been found to be similar in normal and hypersensitive animals injected with either PPD or Hanks' solution alone (Nelson and Boyden, 1963 a).

It is of particular interest that the anti-

coagulants also inhibited delayed skin reactions to PPD in hypersensitive guinea pigs. The inhibition by sodium warfarin or by heparin in large doses was as great as or greater than that caused by cortisone alone in the dose used. Histological studies showed that the diminution in the size of the skin reactions in the warfarin-treated animals was associated with a failure of macrophages to accumulate at the sites, whereas as in the cortisone-treated animals it was associated with a less marked inhibition of macrophage accumulation and a more marked inhibition of polymorph accumulation. In animals treated with both warfarin and cortisone the cellular reaction was grossly diminished and macrophages were almost absent.

The inhibition by anticoagulants of both the macrophage disappearance reaction in the peritoneal cavity and the accumulation of macrophages at the sites of delayed skin reactions suggests that the same response to antigen might form the basis of the two phenomena. The failure of cortisone to inhibit greatly the reaction of peritoneal macrophages, although it inhibits the skin reactions appears to contradict this suggestion. This conflict could be resolved by supposing that the anticoagulants inhibit the reactions of cells in the presence of antigen whereas cortisone inhibits the mobility of the macrophages or reduces

the number available to react with the antigen. Both the experiments reported here and the known ability of hydrocortisone to inhibit the migration of cells in tissue culture (Ketchel, Favour and Sturgis, 1958) are in accord with this interpretation.

These findings may have some practical significance. Certain diseases of man, such as rheumatoid arthritis and systemic lupus erythematosus, may be due to auto-immunity and delayed-type hypersensitivity to autologous tissue antigens may play a larger part than humoral auto-antibody (Waksman, 1962). In these patients treatment with adrenocortical steroids frequently produces beneficial results (Mackay and Burnet, 1962). The experiments described here suggest, however, that anti-coagulants may be more effective, either alone or in combination with cortisone, in preventing the reaction of macrophages to antigen and the accumulation of macrophages at the site of a delayed skin reaction. The efficacy of anticoagulants in the treatment of these diseases may be well worth investigation.

It is not known how the anticoagulants act in inhibiting the macrophage disappearance reaction. The two drugs examined were chosen because their mode of action as anticoagulants is different. Heparin directly inhibits the action of several clotting factors (Factors V, VIII and

IX) (O'Brien, 1958, 1960; Shanberge, Sarelis and Regan, 1959; Niewiarowski and Wegrzynowicz, 1960). Sodium warfarin inhibits the synthesis of Factors II, VII, IX and X (Owren, 1959; Norén and Quick, 1963). Heparin has an anticomplementary as well as an anticoagulant effect, but the results of C' titrations of the sera of warfarin-treated guinea pigs indicated that depression of C' activity was not responsible for the inhibition of the macrophage disappearance reaction in these animals. An overall interference with the clotting process and the formation of fibrin is probably not responsible, as previous experiments had shown that the clumping of macrophages before their disappearance was not due to the presence of fibrin between the cells (Nelson and North, 1963). It seems more likely that the anticoagulants act either by inhibiting the reaction between antigen and the cells of hypersensitive animals or by inhibiting a further reaction consequent upon this. For reasons discussed elsewhere it seems unlikely that the macrophage disappearance reaction is due simply to the attachment of macrophages to each other and to other cells by means of complexes between antigen and cell-bound antibody (Nelson, and North, 1963; Nelson, 1963 c). Furthermore, high concentrations of heparin do not inhibit the uptake of antigen in vitro by the peritoneal cells of

hypersensitive guinea pigs (Nelson and Boyden, 1963 b). The increased stickiness of macrophages, which appears to be responsible for the macrophage disappearance reaction, may thus be an indirect rather than a direct consequence of the reaction of macrophages with antigen. It may be that factors involved in blood coagulation are also required for this change to occur in macrophages. The lack of correlation between the inhibition of the macrophage reaction and the prolongation of clotting time in warfarin treated animals seems to weigh against this. The Thrombotest reagent measures the combined activity of Factors II, VII, IX and X. Depression of only one or two of these factors might be responsible for the inhibition of the macrophage reaction, and might not be accurately reflected by the Thromotest time. If only one factor is involved, this may be Factor IX (Christmas factor) which is both inhibited by heparin and depressed in warfarin-treated animals. Other explanations are possible. The evidence available does not strictly allow a decision whether heparin and warfarin are acting in similar ways. Nor does it strictly allow us to conclude that the antigen reacts directly with macrophages. The affinity for antigen in vitro of the macrophages of hypersensitive guinea pigs suggests, however, that this does happen (Boyden, 1963; Nelson and Boyden, 1963 b). In the

experiments which initiated this investigation it was noted that the serum from clotted undiluted peritoneal exudates induced the disappearance of macrophages when injected into normal guinea pigs. One likely hypothesis is that in the clotting process some substance is liberated which acts on macrophages making them more sticky; and that this process may have some step or steps in common with the reactions which lead to increased stickiness of macrophages of hypersensitive animals injected with antigen. The common step or steps could involve Christmas factor.

It is interesting to note that the synthesis of Factor II and Factor VII, which is inhibited by warfarin, takes place in macrophages (Bujasz, 1954; Slätis, 1958) and that prolonged treatment with other anticoagulants related to coumarin has been found to depress the phagocytic capacity of the macrophages of the RES in humans (Adlercreutz and Pettersson, 1963). The relationship of these findings to the effects of anticoagulants on delayed-type hypersensitivity reactions is not clear.

Other interactions of immunological processes and coagulation mechanisms are known to occur. Complexes of antigen and humoral antibody can accelerate clotting in vitro (Robbins and Stetson, 1959). This effect seems to be mediated by either platelets or blood leucocytes, as

the coagulation of plasma is not accelerated by the complexes. The initiation of blood coagulation in vivo by antigen-antibody complexes (Lee, 1963) may also involve platelets or leucocytes. The acceleration of plasma clotting by bacterial endotoxins in vitro requires the presence of platelets; whether leucocytes can act instead of platelets is not known (Horowitz, Des Prez and Hook, 1962). The possible interrelationships between coagulation factors, and the reactions of antigen with cellular and humoral factors in hypersensitive animals may be of great importance.

Some other observations made in these experiments deserve comment. The slight inhibitory effect of Phenergan on the macrophage disappearance reaction suggests that histamine may play a small part and is compatible with the observation of Pepys (1955) that massive doses of Phenergan inhibited delayed skin reactions to PPD. The failure of reserpine, in the dose used, to inhibit the macrophage disappearance reaction, suggests that serotonin release plays no part in this reaction. Lastly, it is of some interest that the polymorphs appearing in the peritoneal cavity were almost entirely neutrophils; the number of eosinophils in the exudates did not increase significantly in either treated or untreated animals injected with PPD.

SUMMARY

131 I-labelled HSA was added to suspensions of peritoneal cells from normal and hypersensitive guinea pigs. The uptake of antigen by the cells was measured after incubation at 0-5°C or 37°C in the presence or absence of normal guinea pig serum. The highest levels of uptake occurred at 0°C in the absence of serum. In

these conditions cells from animals with Arthus or delayed-type hypersensitivity to HSA took up twice as much antigen as the cells from normal or non-sensitized animals. Cells from animals with mixed Arthus and delayed-type hypersensitivity to HSA, which had been recently skin

PAPER VI.

THE UPTAKE OF ANTIGEN IN VITRO BY PERITONEAL
CELLS OF NORMAL AND HYPERSENSITIVE GUINEA PIGS

Cells from animals with pure Arthus hypersensitivity, recently skin tested, took up only as much antigen as did cells from animals with pure delayed-type hypersensitivity. In none of the animals was there a correlation between the uptake of 131 I-HSA in vitro and the titre of antibodies to HSA or the intensity of the skin reactions to HSA.

SUMMARY

I^{131} -labelled HSA was added to suspensions of peritoneal cells from normal and hypersensitive guinea pigs. The uptake of antigen by the cells was measured after incubation at $0-5^{\circ}\text{C}$ or 37°C in the presence or absence of normal guinea pig serum. The highest levels of uptake occurred at 0°C in the absence of serum. In these conditions cells from animals with Arthus hypersensitivity to HSA took up twice as much antigen as the cells from normal or BCG-vaccinated animals. Cells from animals with mixed Arthus and delayed-type hypersensitivity to HSA, which had been recently skin tested, took up 10 times as much antigen as did cells from normal animals. Cells from animals with mixed Arthus and delayed-type hypersensitivity, which had not been recently skin tested, took up only as much antigen as did cells from animals with pure Arthus hypersensitivity. In none of the animals was there a correlation between the uptake of I^{131} -HSA in vitro and the titre of antibodies to HSA or the intensity of the skin reactions to HSA.

INTRODUCTION

The presence of cell-bound antibody in animals with delayed-type hypersensitivity has frequently been postulated. The existence of such antibody has been inferred from two lines of evidence: (1) the successful passive transfer of delayed-type hypersensitivity to normal animals by means of cells but not by means of serum from hypersensitive animals (e.g., Chase, 1945); (2) the inhibitory effect of antigens on the migration of cells in tissue cultures from hypersensitive animals, an effect apparently not due to humoral antibody (e.g., Rich and Lewis, 1932).

It would be expected that cells possessing this antibody would have a high affinity for the antigen. In animals with tuberculin sensitivity (the classical example of delayed-type hypersensitivity) it has been found that the affinity of cells for tuberculin in vitro or in vivo is not significantly different from that of cells from normal animals (Ritts and Favour, 1955; Turk, 1960; Oort and Turk, 1963). Turk (1960) also measured the uptake of antigen in vitro by lymph node cells of animals with mixed Arthus and delayed-type hypersensitivity to bovine serum albumin (BSA) or bovine gamma globulin (BGG). He found that BSA was specifically taken up by the cells of

BSA-sensitive animals but that BGG was taken up to the same extent by the cells of BGG- and BSA-sensitive animals. The cell suspensions used by Turk were, however, taken from tissue capable of producing antibodies. It is conceivable that some of these cells were plasma cells and took up antigen by virtue of antibody which they had synthesized but had not yet released (Makela and Nossal, 1961). The cell-bound antibody postulated to exist in animals with delayed-type hypersensitivity should be demonstrable on cells which are not actively synthesizing conventional humoral antibody, i.e., cells not of the plasma cell series. The macrophages of peritoneal exudates of guinea pigs with delayed-type hypersensitivity are known both to transfer delayed-type hypersensitivity to normal guinea pigs (Chase, 1945) and to react to injections of antigen by disappearing from the exudates (Nelson and Boyden, 1963 a). They should therefore be suitable for determinations of cellular affinity for antigen. In the experiments reported here the uptake of I^{131} -HSA by peritoneal cells from guinea pigs sensitized to HSA was measured.

ml., dialyzed in the cold against running water for 24 hours and against saline for 24 hours. It was stored at

¹ Referred to as HSA in complete adjuvant and HSA in oil, respectively.

MATERIALS AND METHODS

Animals. Coloured or albino guinea pigs of either sex were used.

Sensitization. The guinea pigs were sensitized by the injection into the hind foot pads of 5 micrograms of human serum albumin (HSA) emulsified in either Freund's complete adjuvant (Difco; containing killed Mycobacterium butyricum) or Freund's incomplete adjuvant (Difco)¹. They were used 4-8 weeks later. Controls comprised normal guinea pigs or guinea pigs sensitized to tuberculin by an intradermal injection of 0.1 mg. BCG 4-8 weeks previously.

Skin tests. The skin tests were performed and read as described previously, with a dose of 5 micrograms HSA (Nelson and Boyden, 1963 a, b).

Radioactive antigen. I¹³¹-labelled HSA was obtained from The Radiochemical Centre, Amersham as a solution containing approximately 20 mg. I¹³¹-HSA per ml. saline. This was diluted in normal saline to a concentration of 2 mg. per ml., dialysed in the cold against running water for 24 hours and against saline for 24 hours. It was stored at

¹Referred to as HSA in complete adjuvant and HSA in oil, respectively.

-20°C. Before use it was thawed and centrifuged at 2,000 g. for 10 min.

Peritoneal cells. The peritoneal cavities were washed out as described previously (Nelson and Boyden, 1963 a). In some guinea pigs exudates had been induced by the intraperitoneal (i.p.) injection of glycogen (0.4 mg. in 10 ml. saline) 4 days previously. In others the cells normally present in the peritoneal cavity were harvested. The total number of cells harvested was calculated from the total cell count and the volume of fluid harvested. Differential cell counts were not made, but it is known from other experiments that the cells comprised approximately 70% macrophages, 25% lymphocytes and 5% polymorphs. More cells were present in glycogen-induced exudates but the distribution of cell types was the same.

Measurement of cellular uptake of I^{131} -HSA. The peritoneal washings containing 10-40 million cells were placed in screw-capped glass tubes. For experiments at 37°C the tubes were lined with paraffin wax to minimize cell adhesion. The cells were centrifuged and washed twice in 12 ml. Hanks' balanced salt solution containing penicillin (50 units per ml.) and streptomycin (100 units per ml.) (HPS). They were resuspended in 1 ml. HPS containing 10 micrograms I^{131} -HSA and, in some experiments,

10% normal guinea pig serum. The suspensions were incubated at the required temperature for $1\frac{1}{2}$ hours, then centrifuged, washed 4 times in 12 ml. HPS, resuspended in 0.5 ml. distilled water, plated on paper discs and allowed to dry. The radioactivity was counted using a Tracerlab Multi-Matic sample changer coupled to a Compu-Matic scaler and an Auto-Computer. The activity of 1 microgram of the I^{131} -HSA was also counted. The antigen uptake was calculated and expressed as millimicrograms I^{131} -HSA per million cells.

Antibody titrations. The tanned sheep cell haemagglutination technique of Boyden (1951) was used.

developed mixed delayed-type and Arthus hypersensitivity (Nelson and Boyden, 1963 b). The cells were incubated with I^{131} -HSA in vitro at 37°C in the presence of normal guinea pig serum. There was very little uptake of antigen by the cells of any of the animals. There was no significant difference between the uptake by cells from animals sensitized with HSA in complete adjuvant and cells from other animals.

At this time other experiments were being carried out in this laboratory on the attachment of sheep erythrocytes to peritoneal macrophages of normal and hypersensitive guinea pigs (Boyden, 1963). The macrophages of guinea pigs sensitized with sheep erythrocytes

RESULTS

Preliminary Experiments

In preliminary experiments peritoneal cells were obtained from normal or BCG-vaccinated guinea pigs, from guinea pigs sensitized with HSA in oil and from guinea pigs sensitized with HSA in complete adjuvant. These animals were not skin tested, but previous experiments had shown that guinea pigs sensitized with HSA in oil developed Arthus hypersensitivity to HSA, whereas those immunized with HSA in complete adjuvant developed mixed delayed-type and Arthus hypersensitivity (Nelson and Boyden, 1963 b). The cells were incubated with I^{131} -HSA in vitro at 37°C in the presence of normal guinea pig serum. There was very little uptake of antigen by the cells of any of the animals. There was no significant difference between the uptake by cells from animals sensitized with HSA in complete adjuvant and cells from other animals.

At this time other experiments were being carried out in this laboratory on the attachment of sheep erythrocytes to peritoneal macrophages of normal and hypersensitive guinea pigs (Boyden, 1963). The macrophages of guinea pigs sensitized with sheep erythrocytes

TABLE 1.

The uptake of I^{131} -HSA under various Conditions
by Guinea Pig Peritoneal Cells

Cells from guinea pigs sensitized with:	Normal guinea pig serum in medium	Uptake of I^{131} -HSA, millimicrograms per 10^6 cells, at temperature:	
		0-5°C	37°C
BCG	None	0.26	0.31
	10%	0.08	0.05
HSA in oil	None	0.99	0.32
	10%	0.82	0.17
HSA in complete adjuvant	None	1.67	0.36
	10%	0.94	0.13

incorporated in complete adjuvant took up large numbers of sheep erythrocytes, whereas macrophages from normal animals or animals sensitized with sheep erythrocytes in oil took up very few. The experiments with sheep erythrocytes were carried out at room temperature and with media containing no guinea pig serum. Further experiments with I^{131} -HSA were therefore undertaken in which macrophages were incubated with the antigen at room temperature and at $0-5^{\circ}\text{C}$. Higher levels of uptake of antigen were observed at the lower temperatures than at 37°C .

In one experiment macrophages were obtained from 4 BCG-vaccinated guinea pigs, 4 guinea pigs sensitized with HSA in oil and 4 guinea pigs sensitized with HSA in complete adjuvant. The cells from the animals in each group were pooled and each pool was divided into 4 equal parts. The cells were incubated for $1\frac{1}{2}$ hours with I^{131} -HSA at $0-5^{\circ}\text{C}$ or at 37°C , with or without 10% normal guinea pig serum. The cells were washed 4 times with HPS, those which had been incubated at $0-5^{\circ}\text{C}$ being kept at 0°C during washing. The uptake of radioactivity was measured as described above. The results of this experiment are shown in Table 1. The uptake of antigen was greatest in each group when the cells were incubated at $0-5^{\circ}\text{C}$ in the absence of serum. In these conditions the cells from

TABLE 2.

The Effect of Recent Skin Tests on the Uptake of
I¹³¹-HSA by Peritoneal Cells of Guinea Pigs with
Delayed-Type Hypersensitivity to HSA

Guinea Pigs sensitized with:	Time since skin test with HSA	Uptake of I ¹³¹ -HSA (millimicrograms/10 ⁶ cells)	
		Individual animals	Mean
Nothing	-	0.28, 0.72	0.50
BCG	-	0.20, 0.14	0.17
HSA in complete adjuvant	14 days	1.12, 0.62	0.87
HSA in complete adjuvant	4 days	3.83, 6.10	4.96

animals sensitized with HSA in complete adjuvant took up more antigen than did the cells from other animals, although the difference was not marked.

In another experiment cells were obtained from 2 normal and 2 BCG-vaccinated guinea pigs and from 4 guinea pigs sensitized with HSA in complete adjuvant. Of the 4 HSA-sensitized guinea pigs, 2 had been skin tested 14 days previously and 2 had been skin tested 4 days previously. The cells from each animal were incubated with I^{131} -HSA at $0-5^{\circ}\text{C}$ in the absence of serum for $1\frac{1}{2}$ hours. They were then washed 4 times in HPS at 0°C . The uptake of I^{131} -HSA was measured. The results are shown in Table 2.

The cells from the animals sensitized with HSA in complete adjuvant and skin tested 4 days earlier took up more than 5 times as much antigen as those from the sensitized animals skin tested 14 days earlier and nearly 10 times as much as those from the normal and BCG-vaccinated animals.

Uptake of Antigen by Peritoneal Cells in vitro in Relation to Skin Reactions and Antibody titres

The results of these experiments suggested that high uptakes of soluble antigen by the peritoneal cells of guinea pigs occurred only when the cells were obtained from animals which had been sensitized with antigen in complete

adjuvant and recently skin tested. To extend the findings in these preliminary experiments a larger experiment was performed. In this experiment the following groups of guinea pigs were used:

- A. 8 normal guinea pigs
- B. 8 guinea pigs sensitized 4 weeks previously with 5 micrograms HSA in oil and skin tested 6 days previously with 5 micrograms HSA
- C. 8 guinea pigs sensitized 4 weeks previously with 5 micrograms HSA in complete adjuvant and not skin tested
- D. 8 guinea pigs sensitized 4 weeks previously with 5 micrograms HSA in complete adjuvant and skin tested 6 days previously with 5 micrograms HSA.

In each group 4 animals received glycogen i.p. 4 days before the experiment. On the day of the experiment the animals were bled and peritoneal cells were obtained. The cells were incubated with I^{131} -HSA in HPS (without normal guinea pig serum) in screw-capped glass tubes rotated at $0-5^{\circ}\text{C}$ for $1\frac{1}{2}$ hours. They were washed 4 times in HPS at 0°C and the uptake of antigen by the cells was determined. The serum was later titrated for antibodies

TABLE 3.

Skin Reactions, Antibody Titres and Uptake of I^{131} -HSA by
Peritoneal Cells of Normal and Hypersensitive Guinea Pigs

Group of Guinea Pigs sensitized with:	No.	Specific increase in skin thickness, mm.			Antibody titre, reciprocal	I^{131} -HSA uptake, millimicrograms per 10^6 cells
		6 hrs.	24 hrs.	48 hrs		
A. Nothing	1*				< 5	0.45
	2*				< 5	0.63
	3*				< 5	0.24
	4*				< 5	0.42
	5				< 5	1.18
	6				< 5	3.32
	7				< 5	0.51
	8				< 5	0.30
						Mean 0.88
B. HSA in oil; skin tested	9*	0.6	0.3	0	20,480	2.34
	10*	0.1	0.2	0	81,920	1.33
	11*	1.0	0.4	0	20,480	0.92
	12*	0.6	0.5	0	10,240	0.99
	13	0.6	0.3	0.1	20,480	2.49
	14	0	0.1	0	81,920	2.68
	15	1.4	0.5	0	5,120	1.92
	16	0.4	0.8	0.4	-	2.64
						Mean 1.91
C. HSA in complete adjuvant; not skin tested	17*				1,280	1.34
	18*				2,560	1.18
	19*				10,240	3.31
	20*				20,480	2.08
	21				40,960	1.96
	22				20,480	1.90
	23				2,560	1.49
	24				5,120	2.51
						Mean 1.97
D. HSA in complete adjuvant; skin tested	25*	0.7	1.5	1.0	40,960	11.68
	26*	0.7	2.8	3.0	20,480	8.23
	27*	0.7	2.3	3.1	81,920	8.15
	28*	0.8	2.0	1.5	81,920	9.56
	29	0.6	2.0	2.2	20,480	2.68
	30	0.5	2.7	2.6	20,480	6.41
	31	1.0	1.5	0.9	10,240	11.97
	32	0.4	3.0	3.6	81,920	9.38
						Mean 8.51

* Injected i.p. with glycogen 4 days previously.

to HSA. The results of the skin tests, antibody titrations and measurements of I^{131} -HSA uptake are shown in Table 3.

The cells from animals sensitized with HSA in complete adjuvant and skin tested before the experiment (Group D) took up much more antigen than did the cells from other animals. These animals had mixed reactions to skin tests, with mild Arthus reactions and strong delayed reactions. There was no correlation between the intensity of the reactions to skin tests and the amount of antigen taken up.

The amounts of antigen taken up by cells from animals of Group C (sensitized with HSA in complete adjuvant but not skin tested) was similar to those taken up by cells from animals of Group B (sensitized with HSA in oil and skin tested). The animals of Group B reacted to skin tests with either mild Arthus reactions (numbers 9, 11, 12, 13, 15) or Jones-Mote reactions (numbers 10, 14 and 16). There was no correlation between the intensity of the skin reaction and the amount of antigen taken up.

The cells from normal animals (Group A) took up half as much antigen as did the cells from animals of Groups B and C and only one tenth as much as did the cells from animals of Group D.

The antibody titres were equally high in Groups

B and D, which had been skin tested. The titres were much lower in Group C, which had not been skin tested. There was no correlation between the antibody titre and the amount of antigen taken up, either in individual animals or between groups of animals.

There was no significant difference in any group between the antigen uptake by cells from animals injected with glycogen and cells from animals not injected with glycogen.

The Effect of Heparin on Antigen Uptake by Cells
in vitro

It has been found that heparin inhibits the disappearance of macrophages from the peritoneal exudates after the injection of antigen into guinea pigs with delayed-type hypersensitivity (Nelson, 1963). It was therefore of interest to see whether heparin inhibited the uptake of antigen by peritoneal cells in vitro.

Peritoneal cells were obtained from 5 guinea pigs sensitized with HSA in complete adjuvant and skin tested 6 days previously. The cells were pooled and divided into 5 aliquots. They were incubated for 10 min. at room temperature with, respectively, 0, 20, 50, 100 and 200 units of heparin. I^{131} -HSA was added and the uptake at 0-5°C measured as in previous experiments. Heparin at all the concentrations tested caused agglutination of the

cells. There was, however, no significant difference in the amount of antigen taken up by the cells incubated with heparin or those incubated without heparin.

DISCUSSION

In these experiments peritoneal cells from guinea pigs sensitized with HSA in Freund's complete adjuvant took up most I^{131} -HSA in vitro when (a) the cells were incubated with antigen in the cold and without serum, and (b) the cells were obtained from animals which had been recently skin tested. In these conditions the uptake was approximately 10 times that of cells from normal animals and approximately 5 times that of cells from animals sensitized with HSA in oil or animals sensitized with HSA in complete adjuvant but not skin tested.

The animals sensitized with HSA in complete adjuvant reacted to skin tests with mixed Arthus and delayed reactions, whereas the animals sensitized with HSA in oil responded with only Arthus or Jones-Mote reactions. A high affinity of peritoneal cells for antigen appeared therefore to be associated with delayed-type hypersensitivity. There was, however, no correlation between the intensity of the skin reaction and the amount of antigen taken up.

The mechanism by which the cells took up antigen was not investigated. It is unlikely that pinocytosis was responsible, as the cells were incubated at 0-5°C, at

which temperature they would be metabolically almost inactive. Opsonization by humoral antibody in the medium is also unlikely, as the cells were thoroughly washed. The release of precipitating antibody into the medium is also unlikely, as the cells were incubated in the cold and we have not observed plasma cells in peritoneal exudates. It seems probable that some substance firmly attached to cells and having a high affinity for antigen was responsible. The experiments described do not permit the conclusion that this was the long postulated cell-bound antibody, as no attempt was made to determine whether it was responsible for, e.g., the ability of peritoneal cells to transfer delayed-type hypersensitivity. Other experiments in this laboratory have shown that the attachment of sheep erythrocytes to peritoneal macrophages from guinea pigs sensitized with sheep erythrocytes in complete adjuvant is mediated by a substance which can be eluted by heating the cells to 56°C. This factor is present in the serum and can become bound to macrophages of normal animals, conferring upon such macrophages the ability to take up sheep erythrocytes (Boyden, 1963). This factor has properties similar to those of cytophilic antibody, as described by Boyden and Sorkin (1960, 1961). It is not known whether a similar factor is responsible for the uptake of I^{131} -HSA.

One difference between the experiments with sheep erythrocytes and the experiments with HSA is that the cells of the guinea pigs sensitized with sheep erythrocytes took up antigen even when the animals had not been skin tested. The reasons for this difference are not clear. It may be that the uptake of soluble proteins by macrophages, even at low temperatures, is a normal event and that a significantly higher uptake due to an immunologically specific cell-associated factor can be detected only when the amount of this factor has been increased by a recent antigenic stimulus. It is known that human erythrocytes and blood leucocytes bind loosely large amounts of globulins (Pirofsky, Cordoba and Imel, 1962; Anderson and Walford, 1960). It is possible that albumins (either native or foreign) behave similarly with guinea pig peritoneal leucocytes. This could also account for the uptake of I^{131} -HSA by the cells from normal animals.

It is interesting to compare the present findings with those of Sorkin, Rhodes and Boyden (1961). They measured the levels of precipitating and cytophilic antibody in the sera and the uptake of I^{131} -HSA by spleen cells from rabbits immunized with HSA. Cells and serum were taken from rabbits injected i.v. once, twice or repeatedly with HSA. The cells were incubated with

I^{131} -HSA at 37°C in the presence of normal rabbit serum. Significant amounts of antigen were taken up only by cells from hyperimmunized rabbits which had received a booster injection of HSA 3-6 days previously; significant amounts of cytophilic antibody were present only in these animals. The uptake of antigen by cells in these experiments was 100-200 millimicrograms per ml. of a 20% suspension of cells. Assuming that such a suspension would contain a minimum of 10^8 cells, the uptake observed by Sorkin et al. was equivalent to a maximum of 1-2 millimicrograms per 10^6 cells - rather less than in the present experiments. Some of the uptake may have been due to cytophilic antibody and some to antibody-producing cells. The differences between these results and the present results may be due to differences in the types of cells present, to differences in the method of immunizing the animals, to differences in the species of animal and/or to differences in the conditions of incubation of the cells with antigen.

The fact that the uptake of antigen was lower at 37°C than at $0-5^{\circ}\text{C}$ may be due either to the ingestion and rapid breakdown of the antigen or to the dissociation of the factor responsible from the cells at the higher temperature. It is known that normal guinea pig peritoneal cells can rapidly degrade complexes formed by

I¹³¹-HSA and rabbit antibody (Sorkin and Boyden, 1959).

The inhibitory effect of normal guinea pig serum on the uptake may also be a result of increased dissociation of the factor from cells in the presence of serum.

The amounts of antigen taken up by cells from animals sensitized with HSA in oil and skin tested were similar to those taken up by cells from animals sensitized with HSA in complete adjuvant but not skin tested. The levels of uptake in these cases were only twice as high as those observed with cells from normal animals. This finding suggests, however, that small amounts of the cell-associated factor responsible may be present in guinea pigs which do not respond to skin tests with delayed skin reactions.

Heparin in large doses in vivo completely inhibited the macrophage disappearance reaction after the injection of antigen into guinea pigs with delayed-type hypersensitivity; smaller doses had less inhibitory effect. Heparin in vitro did not significantly affect the uptake of antigen by peritoneal cells. These results suggest that the inhibitory effect of heparin on the macrophage disappearance reaction in vivo was not due to the dissociation of a cell-associated factor (? cell-bound antibody) from the cells.

The use of particulate antigens for the study of

the reactions of peritoneal macrophages in vitro has certain advantages over the use of labelled soluble antigens, such as I^{131} -HSA. With particulate antigens, such as erythrocytes, no lengthy washing procedures are necessary and the uptake of antigen by cells from animals with delayed-type hypersensitivity appears to be more specific. The experiments described here may, however, provide a partial explanation for the difficulties encountered by other workers (e.g., Turk, 1960) in demonstrating a high specific uptake of antigens in vitro. The explanation may lie in the necessity to incubate cells at low temperatures without serum and the necessity to obtain cells from animals which have recently been skin tested.

D. CONCLUDING DISCUSSION

I. INTRODUCTION

In the Introductory Review (Part A) emphasis was placed on the widespread occurrence of delayed-type hypersensitivity in man and animals and on the dearth of experimental data which could lead to an understanding of its nature. The six preceding papers contain descriptions of some experimental approaches to the problems of delayed-type hypersensitivity. The possible significance of the findings has been discussed briefly therein. In this concluding section the results are summarized and some further interpretations and speculations are offered.

II. THE INDUCTION OF DELAYED-TYPE HYPERSENSITIVITY

It has been noted that delayed-type hypersensitivity occurs after many infections, that it is associated with homograft rejection and experimental auto-immune diseases and that, in combination with Arthus hypersensitivity, it can regularly be induced by the injection of pure protein antigens emulsified with Freund's complete adjuvant. The results of the experiments described in Papers I and II indicated that the skin reactions to pure proteins in guinea pigs sensitized with native or modified proteins emulsified with Freund's incomplete adjuvant (lacking Mycobacteria)

were different from classical delayed reactions. The term Jones-Mote reaction was used to describe reactions which were delayed in onset but which, unlike classical delayed reactions, did not persist for more than a few hours. Jones-Mote reactions usually occurred only in animals skin tested for the first time 7 days after sensitization with antigen in Freund's incomplete adjuvant; animals skin tested for the first time or retested 14 days after sensitization usually responded with pure Arthus reactions.

The nature of Jones-Mote hypersensitivity is not known. It may be a form of hypersensitivity quite different from either Arthus or delayed-type hypersensitivity; or Jones-Mote reactions may depend on humoral antibody similar to that responsible for Arthus reactions and be delayed in onset simply because the production of this antibody is delayed; or they may be very mild delayed reactions. The second hypothesis, that they represent a form of Arthus reaction, would accord well with the temporal and morphological character of the reactions.

The third hypothesis, that Jones-Mote reactions are very mild delayed reactions, resembles the interpretation placed by other workers on their observations of skin reactions in similarly sensitized animals (e.g., Benacerraf and Gell, 1959; Uhr, Salvin and Pappenheimer, 1957; Salvin, 1958).

There is indirect evidence that some of the changes which occur to a marked extent in animals with delayed-type hypersensitivity also occur to a slight extent in animals which do not respond to skin tests with classical delayed reactions. For example, peritoneal cells from guinea pigs sensitized with HSA in complete adjuvant and skin tested took up considerable amounts of antigen when incubated in vitro with I^{131} -HSA. Although much lower, the levels of uptake by cells from animals sensitized with HSA in oil and skin tested were similar to the levels of uptake by cells from animals sensitized with HSA in complete adjuvant but not skin tested; the levels were higher in each case than those observed with cells from normal animals. These findings suggest that the cell-associated factor responsible for antigen uptake was present in animals which did not respond to skin tests with classical delayed reactions (Paper VI). A high serum titre of cytophilic antibody appears to be characteristic of guinea pigs with delayed-type hypersensitivity; but cytophilic antibody may also be present in low titre in the sera of guinea pigs with Arthus hypersensitivity or of rabbits hyperimmunized by repeated intravenous injections of antigen (Boyden, 1963 a, b; Boyden and Sorkin, 1960, 1961). It is conceivable that delayed-type hypersensitivity to pure protein antigens develops in all sensitized animals whatever the mode of

sensitization, but that it is only of very slight degree unless the antigen is incorporated in adjuvants containing Mycobacteria (or substances derived from Mycobacteria).

Even so, it would not necessarily follow that this is the cause of Jones-Mote reactions. Some speculations on the nature of immune responses have been based on the interpretation of Jones-Mote reactions as being manifestations of delayed-type hypersensitivity. In the light of the differences between Jones-Mote and classical delayed reactions, such speculations must be viewed with caution.

The experiments described in Papers I and II gave clear evidence of the effectiveness of adjuvants containing Mycobacteria in promoting the development of delayed-type hypersensitivity to pure protein antigens. The way in which this is brought about is, however, unknown. Experiments directed towards a better understanding of the mode of action of adjuvants in the induction of delayed-type hypersensitivity are required. These might lead in turn to a better understanding of the pathogenesis of certain human diseases, such as systemic lupus erythematosus, rheumatoid arthritis and Hashimoto's thyroiditis, in which delayed-type hypersensitivity to autologous tissue antigens is believed to play a part.

III. THE MECHANISM OF DELAYED-TYPE HYPERSENSITIVITY REACTIONS

The experiments described in Paper III showed that when antigen is injected into guinea pigs with delayed-type hypersensitivity the macrophages form clumps and then disappear almost completely from the exudate. This reaction (the macrophage disappearance reaction) occurs in guinea pigs with pure delayed-type hypersensitivity or with mixed delayed-type and Arthus hypersensitivity, but not in guinea pigs with pure Arthus hypersensitivity. The experiments described in Paper IV strongly suggest that the reaction is due to the adherence of macrophages, in clumps and singly, to the lining of the peritoneal cavity. The reaction was found to be strongly inhibited by anticoagulant drugs (heparin and sodium warfarin), slightly inhibited by cortisone and Phenergan and not inhibited by reserpine. The mechanism by which the cells become more adhesive is not known. There is evidence that strongly suggests that disappearance is not due simply to the attachment of macrophages to each other and to cells lining the peritoneal cavity by virtue of bonds between antigen and cell-bound antibody. Heparin inhibits the macrophage disappearance reaction in vivo but does not significantly affect the uptake of antigen by macrophages in vitro (Paper VI; Boyden, 1963 b). The uptake of antigen in vitro by cells from hypersensitive guinea pigs is

depressed by normal serum; but serum or plasma is present in the peritoneal cavity where the macrophage disappearance reaction takes place. There is other, more tenuous evidence that the macrophages adhere much more readily to foreign surfaces, as well as to tissue surfaces. It has been noted that macrophages harvested from the peritoneal cavities of hypersensitive guinea pigs after the injection of antigen but before the cells have disappeared often adhere very rapidly to the coverslip of a haemocytometer at room temperature; this has not been observed with macrophages from normal guinea pigs or hypersensitive guinea pigs not injected with antigen (unpublished observations). It should be possible to measure this quantitatively and future work along these lines is planned.

It is not known whether the increased adhesiveness is a consequence of a reaction between antigen and cell-bound antibody or a reaction between antigen and humoral antibody. The former hypothesis is the more attractive. The inhibitory effect of anticoagulants on the macrophage disappearance reaction suggests that a co-factor(s) is involved which may be identical with a blood coagulation factor(s), perhaps Christmas Factor. It has been observed that intraperitoneal injection of serum from recently harvested undiluted peritoneal fluid is also followed by the disappearance of macrophages from the exudate. This

suggests that during clotting of this cell rich fluid a factor is released which brings about the same change in macrophages in vivo as does the injection of antigen into guinea pigs with delayed-type hypersensitivity. The role of blood coagulation factors or other co-factors in cellular reactions in delayed-type hypersensitivity requires further investigation.

The macrophage disappearance reaction thus seems to follow an immunologically specific reaction, perhaps at the surface of macrophages and perhaps involving a humoral co-factor(s), as a result of which the macrophages become more adhesive. It seems likely that the increased adhesiveness can also be brought about by non-immunological means and that it is non-specific in that it may be apparent when macrophages are in contact with a foreign surface such as glass. It is possible that the increased adhesiveness is due to a "sticky substance" which can be released as a result of either an immunological reaction or of a non-immunological reaction such as clotting.

Increased adhesiveness of macrophages may play a large part in other manifestations of delayed-type hypersensitivity. The accumulation of macrophages at the site of a delayed skin reaction may occur because the cells become more adhesive when they reach the injection site, where antigen is concentrated. The experiments described

in the preceding papers offer no clue to the mechanism whereby macrophages reach the site in the first place. It may be that they are attracted by a chemotactic mechanism, or it may be that they are in a state of constant circulation through the body in a manner similar to that of lymphocytes (Gowans, 1959). The former hypothesis would be supported by the work of Boughton and Spector (1963). They made counts of the numbers of polymorphs and macrophages appearing at the injection sites at different times after the intradermal injection of tuberculin into hypersensitive guinea pigs. Both polymorphs and macrophages emigrated from the blood vessels, but only the macrophages persisted at the reaction sites. The guinea pigs they used were, however, sensitized with killed tubercle bacilli in oil, a method of sensitization which leads to strong Arthus hypersensitivity and mild delayed-type hypersensitivity. The relevance of Boughton and Spector's findings to the mechanism of pure delayed reactions, such as are observed in animals sensitized with small doses of live tubercle bacilli, is not clear. The slow and progressive increase in size of pure delayed reactions might be better explained on the basis of the second hypothesis above, that macrophages slowly circulating through the tissues are arrested at the site of the reaction. Whatever the way in which these cells reach the reaction site, their

persistence may well be due to increased adhesiveness resulting in their immobilization.

In the increased adhesiveness is due to the release of a "sticky substance" when antigen is injected into animals with delayed-type hypersensitivity, other results can also be explained. Experiments with tritiated thymidine-labelled cells have shown that when guinea pigs are sensitized passively by injections of cells from hypersensitive guinea pigs, the majority of the cells which accumulate at the site of a delayed skin reaction have not originated from the hypersensitive donor (Part A, p. 42). It is possible that these cells accumulate when the "sticky substance" is released after the reaction of antigen with a few cells from the hypersensitive donor.

Similar considerations apply to the accumulation of macrophages in the beds of homografts undergoing rejection and at the sites of tissue damage in auto-immune diseases.

The formation of a tubercle in animals infected with M. tuberculosis (or of similar lesions in other infections) may depend on analogous reactions. Once the infected animal has developed delayed-type hypersensitivity the macrophages may react by becoming more adhesive in the vicinity of bacilli, thereby accumulating locally. They could then be transformed into epithelioid cells or, by fusion, into giant cells. The way in which the infection

is resisted may be immunologically specific or immunologically non-specific. The relationship between delayed-type hypersensitivity and immunity has not been explored in the present study but is obviously of great importance. It is possible that the accumulation of macrophages may be brought about by an immunologically specific reaction but that the subsequent processes whereby the infection is resisted are immunologically non-specific.

Later effects of antigen on the cells of animals with delayed-type hypersensitivity are also important, but have not been studied in the present series of experiments. One such effect is cellular proliferation. Mackaness (1963) and Forbes and Mackaness (1963) observed that, after injection of antigen into hypersensitive mice, the peritoneal cells synthesized DNA (as shown by the incorporation of tritiated thymidine) and later underwent mitosis. Serial observations suggested that some of the cells involved were macrophages and some were lymphocytes which appeared to differentiate into macrophages after division. Somewhat similar findings were reported by Kosunen, Waksman, Flax and Tihen (1963). They noted that tritiated thymidine was incorporated into mononuclear cells at the sites of delayed skin reactions in guinea pigs and rats. The cells which were active appeared to be medium or large lymphocytes,

but it is difficult to be sure of the exact nature of a cell which is about to divide, especially when it is seen in a thin section. It is possible that they were actually macrophages or that they differentiated later into macrophages. Cell proliferation in response to antigenic stimuli in animals with delayed-type hypersensitivity may be an important factor in resistance to infection. Macrophage adherence and proliferation could together greatly increase the density of the macrophages at the site of infection and this in turn may be an important factor in resistance, whether the anti-microbial factors associated with the cells are immunologically specific or non-specific (Mackaness, 1954; Cohn, 1962).

Some of the manifestations of delayed-type hypersensitivity in vitro may also depend on increased adhesiveness of macrophages exposed to antigen. From the time of the original experiments of Rich and Lewis (1932) until the present (e.g., David, El-Askari, Lawrence and Thomas, 1963) many workers have observed that the migration in vitro of cells from animals with delayed-type hypersensitivity is inhibited by antigen; and that inhibition of migration is associated with the death of the cells. This has been attributed to a cytotoxic effect of the antigen on cultured cells. In a careful study, Marks

(1958) showed that the cells died as a result of their failure to leave the explant, especially when cultural conditions were unfavourable. There is no good reason to assume that the primary effect of the antigen in these conditions is a toxic one. It seems possible that the macrophages cultured in the presence of antigen become more "sticky" and adhere firmly to each other and to the walls of the culture vessel. This adhesion could make the migration of the cells a much more difficult and slower process. The possibility was considered above that the increased adhesiveness might be due to the release of a "sticky substance". If this were so, it would explain why migration of normal cells is inhibited when they are cultured with hypersensitive cells and antigen (David et al., 1963; Paas, Flick, Kapral and Rudd, 1961).

An unwillingness to attribute the failure of migration of cells in these conditions to a cytotoxic effect of antigen stemmed from the experiments described in Paper IV. No evidence was obtained that death of or damage to peritoneal macrophages occurred in hypersensitive guinea pigs injected with antigen. In some of these experiments macrophage clumps which had formed in vivo were seen to disperse in vitro, even when the antigen (PPD) was present in the culture medium. These observations seem to

contradict the hypothesis offered, that the inhibition of migration is due to increased adhesiveness of the macrophages. There are, however, marked differences between the conditions of these experiments and of the experiments of other workers. For example, the cells were observed for only 1 hour, whereas inhibition of migration of cells in tissue culture has been reported to take 24-48 hours. The concentration of PPD in our experiments was only 10 micrograms/ml. whereas other workers have found it necessary to use PPD at much higher concentrations (e.g., 100-130 micrograms/ml. in the experiments of Marks). It may be also that the composition of the medium in which the cells are cultured affects their reactivity in the presence of antigen. A co-factor, possibly related to blood coagulation factors, may be necessary for the macrophages to become more sticky in the presence of antigen. The absence of such a factor, which may be unstable, or its presence in only small quantities, could account for the dispersal of the clumps in our experiments. It could also explain the need for a high concentration of antigen and a long period of incubation in the experiments of other workers.

A "cytolytic" effect of antigen on suspended cells from animals with delayed-type hypersensitivity has been reported. This may have a similar basis. In some of these

studies lysis of cells has actually been observed (e.g., Favour, 1947) but in most it has been inferred from the fact that cells disappeared from the suspension (e.g., Feeley and Pickett, 1962). As has been pointed out (Part A, pp48-49) the relationship of these reactions to delayed-type hypersensitivity is not clear. Furthermore, polymorphs and lymphocytes seem to be affected, rather than macrophages. It is, however, possible that the loss of cells from suspension is due to their adherence to the walls of the vessel in which they are contained; this adherence may depend on changes similar to those which macrophages undergo in the course of the macrophage disappearance reaction in vivo.

IV. THE INTERACTION OF CELLULAR AND HUMORAL FACTORS IN DELAYED-TYPE HYPERSENSITIVITY AND HOMOGRAFT REJECTION

It was noted in the Introductory Review that both delayed-type hypersensitivity and the ability to reject homografts in an accelerated fashion were regularly transferable to normal animals by means of cells from actively sensitized donors. It was also noted that some workers had adduced evidence that passive transfer of hypersensitivity with serum was also feasible. Homograft

immunity has been better studied from this point of view than has delayed-type hypersensitivity to bacterial or pure protein antigens. In the case of homograft immunity, the accumulated evidence indicates that in different experimental conditions passively transferred serum from sensitized animals can: (1) have no effect; (2) cause a white graft reaction; (3) prolong the life of the graft by immunological enhancement; (4) cause accelerated rejection of the graft (Part A, p.21). The experiments of Boyden (1963 a, b) suggest that cytophilic antibody is closely associated with delayed-type hypersensitivity in guinea pigs. The differing effects of serum could perhaps be explained in terms of the relative amounts of cytophilic antibody and classical antibody in a particular serum.

Serum which, when passively transferred from homograft sensitive donors, does not affect homografts, may have little or no antibody of either sort specific for graft antigens. The white graft reaction is similar histologically to an Arthus reaction, and the effectiveness of serum in bringing about this type of rejection is thus not difficult to understand.

Serum which contains cytophilic antibody to high titre and other antibodies to low titre may be effective in producing homograft rejection because the cytophilic antibody confers on the macrophages of the recipient the ability to

react with antigen in the bed of the homograft. Serum which is effective in enhancing a homograft in the recipient may contain cytophilic antibody in too low a titre to be effective in sensitizing the recipient's macrophages, but may contain enhancing antibodies in high titre.

In some cases, a particular serum can produce accelerated rejection when injected in large doses and enhancement when injected in small doses (e.g., Kaliss, 1958; Phillips and Stetson, 1963). It may be that cytophilic antibody is ineffective unless it is injected in large doses, whereas enhancing antibody is effective in much smaller doses.

The experiments described in this Thesis also suggest two possible ways in which enhancing antibodies may act. (1) It is possible that they react with antigen being liberated from the homograft before the antigen can react with macrophages; the macrophages would then not undergo the change towards increased adhesiveness which, it has been postulated, leads to their accumulation in the homograft bed. (2) It is possible that the reaction of homograft antigens with enhancing antibodies and (probably) complement leads to the liberation of heparin from mast cells and/or platelets. The heparin released could prevent the increased adhesiveness of macrophages which have reacted

with antigen; this could also prevent the accumulation of macrophages in the bed of the graft. Both these hypotheses would be disproved if it were shown that enhanced grafts survive despite the accumulation of macrophages in the bed; but histological studies on this point are lacking (Part A, pp. 56-58).

Similar interactions may also help to explain the different effects of bacterial endotoxins (Part A, pp. 23-27).

V. CONCLUSION

In the Introduction to this Thesis four major problems in the study of delayed-type hypersensitivity were cited. Partial answers to these are now available.

(1) The nature of the changes which lead to altered reactivity of cells in delayed-type hypersensitivity. The experiments described in Paper VI, when considered together with those of Boyden (1963 a, b), suggest that at least one such change is that antibody, having the properties of cytophilic antibody, is attached to macrophages.

(2) The nature of the forces attracting cells to and holding them at the sites of delayed skin reactions, in the beds of homografts undergoing rejection and at sites of tissue damage in auto-immune diseases. The experiments described in Papers III, IV and V suggest that the accumulation of macrophages may depend on their increased

adhesiveness in the presence of antigen; and that this change may require the participation of co-factor(s) possibly related to blood clotting factors.

(3) The mechanisms underlying the induction of delayed-type hypersensitivity as opposed to humoral antibody formation and Arthus hypersensitivity. This remains a problem. The experiments described in Papers I and II indicate that classical delayed-type hypersensitivity to pure protein antigens could regularly be induced in guinea pigs only when the sensitizing injection was incorporated in adjuvant containing Mycobacteria. In animals sensitized in this way delayed-type hypersensitivity was usually accompanied by Arthus hypersensitivity, although modification of the antigen, for example, by complexing it with a hapten or with antibody, delayed the onset of Arthus hypersensitivity.

(4) The absence of a reliable test for delayed-type hypersensitivity other than the skin test. This too remains a problem. The experiments described in Paper VI showed that high levels of uptake of soluble antigen by cells in vitro occurred only in very limited experimental conditions; this is unlikely to provide an answer. On the other hand, the affinity of macrophages for particulate antigens in vitro may be extremely useful. It may also

be possible to devise an in vitro test based on the clumping and increased adhesiveness of macrophages which seem to be responsible for the macrophage disappearance reaction in vivo.

Dr. S.V. Boyden for his help

throughout the course of this work

Thanks are also due to

the preparation of the histological

for the histological preparation

A. Rumph, Mr. J. Conrad and

of the Animal Breeding Station

and care of the animals. Mr. R. H. H.

Roberts, Miss Margaret Fennell and

Baris, for technical assistance. The

staff of the Photography Department

material; my wife, for drawing the

Judith O'Connor for the preparation

ACKNOWLEDGEMENTS

I wish to acknowledge my indebtedness to Dr. S.V. Boyden for his advice and encouragement throughout the course of this work.

Thanks are also due to: Mr. R.J. North, for the preparation of electron micrographs; Mr. R. Hill, for the histological preparations; Mr. N. Brown, Mr. A. Rumph, Mr. J. Gozzard and other members of the staff of the Animal Breeding Establishment, for the supply and care of the animals; Mrs. Diana Heath, Miss Leone Roberts, Miss Margaret Percival and Mrs. Elizabeth Barta, for technical assistance; Mr. V. Paral and the staff of the Photography Department for the photographic material; my wife, for drawing the graphs; and Miss Judith O'Connor for the preparation of the typescript.

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